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THE INFLUENCE OF SPECIFIC ANTISERA AND UNHEATED GUINEA PIG SERUM ON THE PATHOGENICITY OF »RUNDE« VIRUS FOR MICE

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Traavik T The influence of specific antisera and unheated guinea pig serum on the pathogenicity of »Runde« virus for mice Acta path microbiol scand Sect B 87 1-8 1979

Antisera from various animal species containing antibodies to »Runde« virus were not able to neutralize virus infection in newborn mice the outcome of which is an acute fatal CNS disease There was, however one noticeable exception Mixtures of virus and hyperimmune mouse serum or ascitic fluid inoculated intracerebrally into newborn mice resulted in a persistent infection and a chronic disease which had previously only been recognized in 2 to 3 week-old mice inoculated with »Runde« virus A serum pool from persistently infected mice had the same effect though this was less pronounced The addition of unheated guinea pig serum to the virus hyperimmune serum mixtures reinforced the tendency to persistence and chronic disease and unheated guinea pig serum alone modified the infection in the same way The results suggest an immunological basis for the virus persistence and chronic disease in suckling mice

Key words Virus antibody mixtures non neutralization chronic disease virus persistence

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»Runde« virus is probably a »new« arbovirus isolated from the seabird tick *Ixodes uriae* (17) Its morphology resembles that of coronavirus No antigenic relationship to avian infectious bronchitis (AIB) virus or major arbovirus groups has been found

This virus causes acute encephalitis in baby mice after intracerebral (ic) injection In 2 week-old mice a persistent infection with chronic disease may develop The virus persists in the brain for up to 150 days despite high antibody titres (20)

The present article reports experiments which demonstrate the inability of antisera from various sources to neutralize the acute disease and the brain multiplication of virus in baby mice

MATERIALS AND METHODS

Virus Strain

The origin and isolation histories of the two »Runde« virus strains Ru E81 and Ru E85 have been given in

was used as a 20 % brain suspension which contained approximately $10^{6.5}$ BMLD$_{50}$ (baby mouse lethal doses) per ml while the infected BHK medium contained approx $10^{6.7}$ BMLD$_{50}$ PBS pH 7.4 with 0.8 % bovine albumine (APBS) was used for virus dilutions

Mouse Strain

The experiments were performed with baby mouse litters of the strain Bom NMRI (SPF) kept and bred at the Department of Laboratory Animals National

Institute of Public Health Oslo Most of the litters consisted of eight baby mice aged 1–3 days In addition some litters aged 14 and 21 days were used due to the tendency to develop persistent infection at that age (20)

Antibody Source

a A pool of sera from 20 adult mice given five intraperitoneal injections as described previously (17) (hyperimmune mouse serum) The pool had titres of 1280 512 and 1024 by HAI (haemagglutination inhibition) CFT (complement fixation test) and IEOP (immunoelectroosmophoresis) respectively

b Pooled ascitic fluids from the animals which had the same antibody titres as the serum pool (hyperimmune mouse ascites)

c Pooled sera from 10 persistently infected mice samples taken 20–40 days p i (chronic mouse) This pool had titres of 160 64 and 64 by HAI CFT and IEOP

d Sera from a technician accidentally exposed to an infected syringe while inoculating mice The first serum (early human) was taken two weeks after exposure and had titres of 10 < 5 and 4 (HAI CFT and IEOP) The second serum (late human) was taken four weeks p i and had titres of 10 8 8 The preinoculation serum contained no antibodies by the methods employed No disease was observed in connection with this accident

e Pools of sera from chickens inoculated subcutaneously with 100–1000 $BMLD_{50}$ of »Runde« virus during the second day after hatching The first pool (early chicken) was from five chickens taken on day 7 p i this had a titre of 20 in HAI but no detectable antibodies in CFT and IEOP The second pool (late chicken) was sera from three chickens taken on day 14 p i The titres were 80 by HAI < 5 by CFT and 16 by IEOP

f A pool of sera from four guinea pigs given four weekly subcutaneous injections of BHK virus (approx 10^4 $BMLD_{50}$) This pool (hyperimmune guinea pig) had titres of 640 in HAI 256 in CFT and 512 in IEOP

g A fowl antiserum to AIB virus supplied by the National Institute of Veterinary Medicine Oslo the neutralization index (NI) was 6 1 It did not react with »Runde« virus in HAI CFT or IEOP

h Sera negative for antibodies to Runde virus by the serological methods used were available for all species These were used as control sera in the neutralization tests

All antisera were inactivated at 56°C for 30 minutes Mouse antisera were absorbed with BHK cells and all others with both cells and uninfected mouse brains proir to use This was also the case with the guinea pig serum No reaction with the control antigens was seen in CHI or IEOP for any of the sera Details of the adaptation of serological methods to »Runde« virus have been given elsewhere (17 18 19)

Virus Neutralization Tests in Mice

Constant virus varying serum dose In these experiments the final inoculum was calculated to contain approximately 100 $BMLD_{50}$ of »Runde« virus per baby mouse Virus and antisera were diluted in APBS with the addition of antibiotics In the initial experiments aimed at evaluating the neutralizing capacity of all the listed sera sera dilutions 1/10 and 1/100 were used In the later experiments focused on the mouse hyperimmune serum dilutions 1/2 1/10 1/100 and 1/1000 were used Samples and buffer were kept on ice during the dilution procedures All virus serum mixtures were made in duplicate After incubation for 30 min at 37°C half of the tubes received unheated guinea pig serum while the other half received an equal volume of APBS The guinea pig serum was added in a concentration sufficient to give approximately five complement doses as calculated from complement haemolysis tests The controls included control titrated virus negative serum and guinea pig serum preheated at 56°C for 30 minutes Each mouse received 0 02 ml intracerebrally

Constant serum varying virus dose In these experiments sera were diluted 1/2 and mixed with virus doses calculated to contain approximately 4 3 2 and 1 $\log_{10}$units $BMLD_{50}$ in the final inoculum

The procedures for handling the different virus serum mixtures the addition of unheated and heated guinea pig serum and the mode of inoculation were as described above

In addition to negative serum virus mixtures and control titrated virus preparations controls with uninfected mouse brains or cell culture media instead of virus were used in the neutralization tests

Re isolation of Virus from Inoculated Mice

Brains were harvested after homogenization and inoculation of suspensions ic into new baby litters as described previously (17) Reisolated virus was identified by the serological methods used in this study and performed as reported elsewhere (17 20)

RESULTS

The Deficient Neutralizing Capacity of Antisera

None of the antisera employed was able to completely neutralize »Runde« virus infectivity in mice (Table 1) Acute disease developed although the incubation time was 1–2 days longer when antisera had been added As a rule all mice were moribund or dead by the 10th day p i However for the hyperimmune mouse serum and hyperimmune ascites a special trend was noted By the 10th day about two thirds of the mice were subacutely ill while the rest seemed to be unaffected By the 20th day p i all animals showed signs of chronic disease The serum pool form persistently infected mice seemed to have the same effect though to a lesser extent No differences between M and BHK virus were recorded

TABLE 1 »Neutralizing« Capacity of Various Antisera to »Runde« virus Mixtures of $10^{2.3}$BMLD$_{50}$ of Ru E81 M Virus and Antisera at the Indicated Dilutions were Inoculated ic into One-day old Mice Two Litters of Six Baby Mice per Serum Dilution

Serum	Dilution	First symptoms (day p i)	Day 10	Day 20	Mortality
Early chick	1/10	7	10 dead 2 moribund	–	100%
	1/100	7	11 dead 1 moribund	–	100%
Late chick	1/10	8	9 dead 3 moribund	–	100%
	1/100	7	11 dead 1 moribund	–	100%
Guinea pig	1/10	8	8 dead 4 moribund	–	100%
	1/100	7	11 dead 1 moribund	–	100%
Chronic mouse	1/10	9	10 subacute 2 asympt	10 dead 2 chron.	83%
	1/100	7	5 dead 3 moribund 2 subacute 2 asympt	11 dead 1 chron	92%
Hyperimmune ascites	1/10	10	8 subacute 4 asympt	12 chron	0%
	1/100	8	10 subacute 2 asympt	6 dead 6 chron	50%
Hyperimmune serum	1/10	10	9 subacute 3 asympt.	12 chron	0%
	1/100	8	10 subacute 2 asympt	5 dead 7 chron	42%
Early human	1/10	7	10 dead 2 moribund	–	100%
	1/100	7	12 dead	–	100%
Late human	1/10	8	4 dead 5 moribund 3 ill	–	100%
	1/100	7	8 dead 4 moribund		
Controls[a]	1/10	6	All dead	–	100%

[a] Include virus control negative sera from the species implyed and an antiserum to avian infectious bronchitis virus

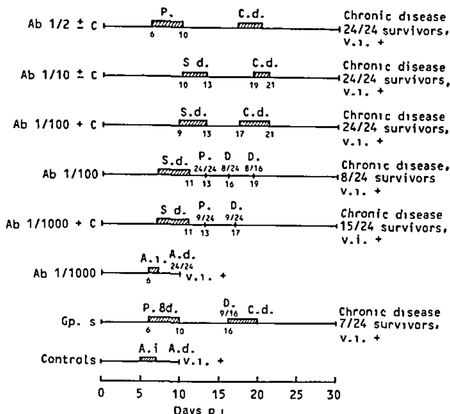


Fig 1 Intracerebral inoculation of baby mice with a constant dose of »Runde« virus ($10^{2.3}$ $BMLD_{50}$) combined with hyperimmune serum in various dilutions and unheated guinea pig serum The number of days after inoculation is stated on the abscissa
(Ab 1/2 etc Dilution of hyperimmune serum used
C and *Gp s* Unheated guinea pig serum
Controls »Runde« virus alone and in combinations with normal mouse serum, heated guinea pig serum, etc
A i Acutely ill *A d* All dead *D* Dead *P* Paresis *S d* Subacute disease *C d* Chronic disease *V i* Virus isolation
The fractions give the number of animals with the symptoms indicated

Mixtures of »Runde« Virus and Hyperimmune Mouse Serum

In the main experiments the effects of mixtures of Ru E81 virus and hyperimmune mouse sera were studied more thoroughly Fig 1 summarizes the results with constant M virus ($10^{2.3}$ $BMLD_{50}$) and varying serum doses Antiserum diluted 1/1000 had no effect on the clinical course All mice contracted an acute illness and the survival time was identical with that of the controls, the only difference being the one day longer incubation time with antiserum At dilution 1/100 the clinical disease was more protracted, with subacute disease and paresis as the main features One third of the mice survived the infection and showed clinical symptoms resembling those of the chronic disease described previously (20) for 2-week-old mice inoculated with »Runde« virus With antiserum diluted 1/10, the protracted course was even more pronounced, and all mice survived with chronic disease At dilution 1/2 the trend was broken, since symptoms commenced already on day 6 p i However, eventually all mice displayed chronic disease and all survived until day 40 p i

The experiments with constant serum and varying virus doses are shown in Table 2 The addition of antibodies influenced the clinical course and the mortality even with the highest virus dose employed In all groups the survivors had chronic disease This was never seen in the virus controls, the mouse brain controls or the negative serum controls

The results for BHK virus (not shown in Fig 1 and Table 2) were essentially the same as for M virus

Influence of Guinea Pig Serum

Heated guinea pig serum (56°C for 30 minutes) did not influence the results The influence of unheated guinea pig serum was unexpected, but nevertheless convincing (Fig 1, Table 2) In mouse

TABLE 2 »Neutralizing« Effects of Hyperimmune Serum (Mouse) and Unheated Guinea Pig Serum on Different Doses of »Runde« E81 M Virus. Twenty four Newborn Mice Infected Intracerebrally

	Status at day p.i.						
Inoculum[a]	5	10	15	20	30	Mortality	Virus isolation
E81 4 logs[b]	3 dead	All dead				100%	NT[c]
Do + Ab[d]	Asympt	Acute paresis	12 dead 9 ill 3 asympt	18 dead 6 chron	6 chron	75%	+ day 40
Do + gps[e]	Symptoms	All dead				100%	NT
Do + Ab + gps	Asympt.	Paresis	6 dead 12 ill 6 asumpt	14 dead 8 ill 2 asympt.	10 chron	58%	+ day 40
E81 3 logs	2 dead	All dead				100%	NT
Do + Ab	Asympt.	Acute paresis	Subacute 9 paresis	9 dead 15 ill	15 chron	38%	+ day 40
Do + gps	Sympt	All dead				100%	NT
Do + Ab + gps	Asumpt.	Slight paresis 1 dead	Subac. 10 paresis	4 dead 18 ill 2 asympt.	18 chron 2 asympt.	17%	NT
E81 2 logs	Slight sympt.	20 dead 4 moribund				100%	+ day 10
Do + Ab	Asympt.	Paresis	chron	chron.	24 chron	0%	+ day 40
Do + gps	Asympt	8 dead 14 ill	14 dead 10 subac	15 dead 9 subac.	9 chron	63%	+ day 40
Do + Ab + gps	Asympt	Slight sympt.	chron	chron	24 chron	0%	+ day 40
E81 1 log	Asympt.	12 dead 7 ill/morib 5 asympt	20 dead 4 asympt.	4 asympt.	4 asympt.	83%	+ day 10 − day 40
Do + Ab	Asympt	Asympt	Slight sympt.	chron	24 chron	0%	+ day 40
Do + gps	Asympt.	Some with paresis	chron.	chron.	24 chron	0%	+ day 40
Do + Ab + gps	Asympt.	Asympt.	Some chron	chron	24 chron	0%	+ day 40

[a] A serum pool from uninfected mice was used as control with all virus doses. Heated instead of unheated guinea pig serum was used with virus doses 3 and 2 logs. These preparations did not affect the 100% mortality rates. Uninfected mouse brain suspensions mixed with antiserum and guinea pig serum did not produce any deaths.
[b] Final virus dose received by each baby mouse given in $\log_{10}$ BMLD$_{50}$
[c] Not tested
[d] Antibody: Hyperimmune mouse serum

litters inoculated with mixtures of virus and guinea pig serum, the mortality was reduced and the survivors developed chronic disease When guinea pig serum was added to mixtures of virus and antiserum, the ability of antiserum to modify the clinical course was reinforced

Experiments in 14 and 21 Day-old-Mice

These animals were inoculated with mixtures of $10^{2.3}$ $BMLD_{50}$ virus and hyperimmune mouse serum diluted 1/2 with or without the addition of unheated guinea pig serum As shown in Table 3, the antiserum or guinea pig serum did not alter the clinical course in these animals

Reisolation of Virus

The failure of the hyperimmune serum to neutralize Ru E81 infectivity was further illustrated by the reisolation of virus from both acutely and chronically diseased mice (Fig 1, Table 2, Table 3)

Extended In Vitro *Incubation Time for Mixtures*

Since we realized that the *in vitro* incubation time of inoculation mixtures had been rather short some experiments were repeated in order to evaluate the effect of an extension In this case virus suspension antiserum dilution and the virus antiserum mixture were prepared in the afternoon and left overnight (appeoximately 18 hours) at + 4C A fresh virus antiserum mixture was made the next morning All tubes were then incubated at 37°C, and unheated guinea pig serum was added as described earlier The prolonged incubation time did not affect the baby mouse pathogenicity, and the results were nearly identical with those obtained in the main experiments

DISCUSSION

The present studies have shown that antisera from different animal species were not able to neutralize the pathogenicity of this virus for newborn mice Since »Runde« virus produces persistent infections

TABLE 3 *Influence of Hyperimmune Mouse Serum and Unheated Guinea Pig Serum on the Course of Ru E81 Infection in 14 and 21 Days Old Mice 16 Animals per Inoculum*

Age of mice	Inoculum	Status at day p i: 10	12	16	30	Virus reisolation
14 days	E81[a] + ums[b]	Asympt	Slight sympt	chron	16 chron	+ day 40
	Do + Ab[c]	Asympt	Slight sympt	chron	1 dead 15 chron	+ day 40
	Do + gps[d]	Asympt	Slight sympt	chron	1 dead 15 chron	+ day 40
	Do + Ab + gps	Asympt	Slight sympt	chron	16 chron	+ day 40
21 days	E81 + uns	Asympt	Asympt	Slight sympt	16 chron	+ day 40
	Do + Ab	Asympt [e]	Asympt	Slight sympt	15 chron	+ day 40
	Do + gps	Asympt	Asympt	Slight sympt	16 chron	+ day 40
	Do + Ab + gps	Asympt	Asympt	Slight sympt	16 chron	+ day 40

[a] $10^{2.3}$ $BMLD_{50}$ (baby mouse lethal doses)
[b] Serum pool from uninfected control mice
[c] Hyperimmune mouse serum diluted ½
[d] Unheated guineapig serum
[e] One mouse died on day 2 p i

in the brains of older suckling mice (20) and blockage or failure of the organism to produce neutralizing antibodies has been proposed as one possible factor in the pathogenesis of persistent infections (8) this is an interesting *in vivo* finding Relative or absolute failure blockage or suppression of neutralizing antibodies have been recognized in other persistent infections e g LCM in mice (9) and Visna maedi in sheep (16) As regards the latter syndrome neutralizing activity can be detected only late in the infection and then only after an *in vitro* incubation period of 48 hours (5) In our experiments no difference was found between an incubation period of 1 and 18 hours The possibility of an »antigenic drift« within the infected animal as has been proposed for Visna maedi (5) is a fascinating explanation for the ability of a persistent virus infection to escape the humoral immunity

The only antibody preparations which showed any influence on the »Runde« virus infectivity in newborn mice were from hyperimmunized or persistently infected mice The virus was not neutralized but the clinical infection was changed and virus persisted in the infected brains These findings indicate a direct part played by the immune system in the different age-expression of »Runde« virus infection in mice The only studies which have indicated an immuno-dependent age resistance to virus infections have been with mumps virus in mice (11 12) The development of chronic instead of acute disease in newborn mice inoculated with virus hyperimmune serum mixtures points to a possible primary role played by immune-complexes also for the chronic disease in older animals (20) Possibly phagocytosing cells with Fc receptors which set up an alternative infection cycle might be involved (10) The immaturity of the lymphoid apparatus of the new born mouse (3 13) and the non myelinization of the newborn mouse brain may provide factors predisposing for acute infection (4)

As regards the part played by the unheated guineapig serum it is well known that »labile serum factors« (which are usually found to be complement) are able to reinforce antibody mediated neutralization of viruses (2 7 10 14) The mechanisms involved may be simple contribution of bulk (1) around the complex agglutination via a C3 receptor in the complex (10 21) virolysis (1) or direct neutralization of virus by complement alone as has been suggested for VSV by *Oldstone* (10) Our results with mixtures of »Runde« virus and unheated guinea pig serum seem to support the assumptions of *Oldstone*

Finally (though this has not been examined in this study) mention will be made of the suggested roles of suppressor T-cells (15) and defective interfering particles (6) for the development of persistent virus infections These factors might also contribute to the different age-expression of »Runde« virus infections in mice (17 20)

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ANTIBODIES TO TICK-BORNE ENCEPHALITIS VIRUS IN HUMAN SERA FROM THE WESTERN COAST OF NORWAY

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Traavik T Antibodies to tick-borne encephalitis virus in human sera from the western coast of Norway Acta path microbiol scand Sect B 87 9–13 1979

Sera from 341 individuals living in the distribution area of the tick *Ixodes recinus* were tested for tick borne encephalitis (TBE) antibodies by HAI and gel diffusion Kaolin treatment was unreliable for the removal of non-specific HAI inhibitors Seven sera positive after this treatment were shown to be negative after acetone extraction/flotation centrifugation The antibody prevalence rate was 19 6 % Seventy-one % of the sera had titres ≥ 40 The prevalence rate decreased with age Some sera with low HAI titres could be confirmed by a sensitive Ouchterlony technique while some with high titres could not even after ten fold concentration Clinical information obtained retrospectively regarding patients with high antibody titres revealed some cases consistent with a TBE virus infection Antibody prevalence rates indicate that TBE virus is more active than Uukuniemi and Kemorovo group virus in tick infested areas Mixed foci of these viruses have been indicated by serological findings and virus isolations

Key words Antibody prevalence non specific inhibitors clinical disease age distribution

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Received 14 ix 77 Accepted 11 vii 78

Publications indicating the existence of tick borne arboviruses in Norway have been available for years (4 12 21) A restricted serolog[illegible]
(24 [illegible]
lite[illegible]
Ixodes ricinus a well known tick vector of this virus In 1976 five virus strains antigenically related to the TBE virus complex were isolated from *I recinus* (27)

The present study presents results emerging from a serological survey of material from humans living in or near *I ricinus* invaded areas on the Western coast of Norway [illegible]

MATERIALS AND METHODS

Virus Antigens and Control Sera

TBE virus (Hypr strain) was provided by the Institute of Virology Slovak Academy of Sciences Bratislava, as a 10% lyophilized baby mouse brain suspension from passage 63 Sucrose acetone (SA) extracted haem[illegible]

a verified case of tick borne encephalitis and a rabbit immune serum of own production (26)

Human and rabbit sera were used as negative controls

Human Sera for Antibody Sreening

A serum panel was selected from the files of the Department of Microbiology The Gade Institute Bergen Norway The donors comprised patients examined for all kinds of viral diseases The only criterion for inclusion in the panel was that the patients should live within the distribution area of *I recinus* Efforts were made to match the panel with respect to age and sex distribution Sera submitted from 341 persons in 1973 and 1974 were finally included These sera have been screened earlier for antibodies to the Norwegian Uukuniemi virus strain By E50 (25) and some of them also to Tribec virus (unpublished)

Pretreatment of Sera

Kaolin absorption of non specific HAI inhibitors was carried out according to *Clarke & Casals* (7) In order to test the adequacy of this method sera found positive in HAI *after kaolin treatment were extracted with acetone* (7 11) and retested for HAI antibodies

Separation of Antibodies and Non specific Inhibitors

Flotation centrifugation in NaBr was performed according to *Blom & Haukenes* (3) employing a Beckman L 350 centrifuge with rotor Sw 50 1

Haemagglutination Inhibition Test

HAI was performed essentially as described by Clarke & Casals (7) We used microtitration equipment chicken erythrocytes and four HA units at pH 6 2 and 4° C Following kaolin treatment the sera were screened at dilutions 1 10 and 1 40 Positive sera were titrated *in duplo*

Immunodiffusion

CHI (23) was performed with selected sera for specificity control of HAI reactions Optimal working conditions were determined by checkerboard titrations of TBE antigen against the positive control sera Some positive HAI reactions could not be confirmed directly and we tried to concentrate sera 10 times by Lyphogel (Gelman) (1) The small volumes avaiable of some sera *prohibited the universal use of this procedure Further* specificity control in CHI was achieved by absorption of positive sera with TBE and control antigens

Correlation to Clinical Disease

No clinical information was available beforehand concerning the patients Later we received information recorded at the Gade Institute for patients with HAI titres ≥ 320 (after kaolin treatment) and for all seropositive patients hospitalized at the Neurological Department *Haukeland Hospital Bergen*

RESULTS

Removal of Non specific Inhibitors

A total of 79 sera showed HAI activity at titres ≥ 10 after kaolin treatment Seven of these sera were negative after acetone extraction four with titre 10 two with titre 20 and 1 with titre 40 Flotation centrifugation of these kaolin treated sera showed HAI activity in the lipoprotein fraction only and after acetone-extraction no activity was found For some of the other sera there was a drop in titre after acetone as compared with kaolin treatment Flotation centrifugation proved this to be due to residual lipoproteins after kaolin treatment while acetone seemed to remove non specific inhibitors completely These results are illustrated in Fig 1

Overall Sreening Results

Seventy two sera from 67 patients had HAI antibodies to TBE virus in titres ≥ 10 after acetone-extraction i e the antibody prevalence rate was 19 6% (67/341) Serum pairs were tested for five patients with identical titres in both sera Geographical locations and proportions of seropositive patients submitted by hospitals/physicians are shown in Fig 2 Sixty three % of the positive sera had titres ≥ 40 and 17% had ≥ 320 The geometric mean titre of the HAI positive sera was 53

Sex and Age Distribution

The prevalence of *seropositive males was 23%* (38/163) and 16% (29/178) females The diffe

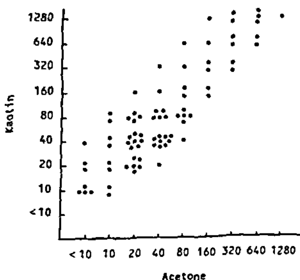


Fig 1 Comparison of HAI titres after kaolin and acetone treatment of 79 sera found positive in screenings following kaolin treatment

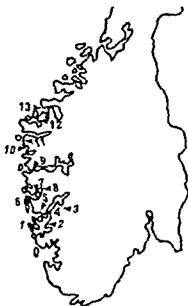


Fig 2 Outline of the southern Norway Locations of physic ans/hosp tals submitting sera from seropos tive pat ents Figures in brackets are numbers of TBE seropos tive/number tested

1 Stord (18/71)
2 Husnes (1/1)
3 Odda (1/10)
4 Fusa (2/3)
5 Tysse (2/3)
6 Bergen (12/45)
7 Fotlandsvåg (1/1)
8 Straumsgrend (4/14)
9 Lavik (1/3)
10 Florø (4/33)
11 Svelgen (3/10)
12 Volda (2/7)
13 Ålesund (16/44)

rence is not significant according to a chi square test with Yates correction for discontinuity (19) How ever there is a decline in the prevalence dependent on age The difference between the age groups 20–30 years and 60–70 years was significant ($p < 0\ 05$) These results are illustrated in Fig 3

Gel D ffusion

Table 1 shows the CHI results for sera of various HAI titres There was no clear-cut relationship between HAI titre and reactivity in CHI Absorption with TBE and control antigens showed all precipitin reactions to be specific

Clinical Evaluation

We obtained clinical information about patients with HAI titres ≥ 160 One of these had a diagnostic rise in titre to *M pneumoniae* Another with a diagnosis of pneumonia had cold agglutinins in serum No diagnosis of virus infection was made for any of the patients

Ten of these patients had symptoms which *may* have been due to TBE virus infection encephalitis polyradiculitis with paresis myalgi and fever The diagnosis doubtful multiple sclerosis was made in two cases

Antibodies to Other Tick Borne Arboviruses

Some of the TBE seropositive patients had antibodies to another probably *I rici nus* transmitted virus Two had antibodies to the Norwegian Uukuniemi strain By E50 (25) and three were seropositive for Tribec virus (Orbivirus Kemorovo group) (unpublished) None of the sera had antibodies to all three virus

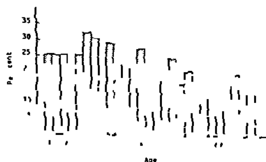


Fig 3 Antibody prevalence rates in various age groups
Dashes Males
Dots Females
Open columns Total

TABLE 1 *Exam nation of HAI Positive Sera by Gel D ffus on*

HAI titre	Number tested	Number of CHI[a] positive sera Direct	Concentrated × 10
10	5	0	0[b]
20	12	4	5
40–160	16	11	13
≥ 320	8	7	8
Total	41	22	26

[a] Closed hexagon immunodiffusion
[b] One serum gave a very faint precipitation line but the identity could not be decided

DISCUSSION

A relatively high proportion of persons with antibodies to TBE has been found among unselected patients representing an average of the cases handled by the regional virus laboratory serving the parts of Norway most heavily invaded by *I ricinus* The average antibody prevalence rate (≈ 20%) runs parallel with the rate for cattle recognized earlier in the same parts of the country (24) The rate seems lower in the endemic TBE areas in Finland (18) Investigations on the tick infested Danish island Bornholm (9) revealed only 1–2% seropositives to TBE among the unselected residents as opposed to 30% among forest workers supposedly exposed frequently to ticks The antibody prevalence in the age group 20–30 years in our patients is exactly the same as that found for Danish forestry workers We have not found publications on antibody screenings of unselected humans in Sweden but the very high rate of bovine reactors (43%) in southeastern Sweden gives the impression of strong virus foci (31) Human CNS disease due to TBE is well documented in both Sweden (20 30) and Finland (14 17 32) It would seem that Norway has active TBE foci of at least the same potency as in the endemic parts of the neighbouring countries Serological screenings of humans and cattle (24) have shown that coastal Norway from the southern end of the country and northwards to Møre and Romsdal county can be considered an endemic TBE area This represents only part of the distribution area of *I ricinus* which stretches from the Swedish border in the south-east almost up to the Arctic Circle in Nordland county (22 27)

The significant difference in age distribution for seropositives in our material is in contrast to the situations reported from Finland (18) and Czechoslovakia (10)

The failure to confirm even some strong HAI reactions by gel diffusion may indicate the existence of related (perhaps mosquito borne) flavivirus in the areas investigated (6 16) Precipitating antibodies seem to be more type specific than the group reacting HAI antibodies (2 5 13 15 25) On the other hand precipitating antibodies are thought to be more short lived than HAI antibodies (2) This may however be a question of sensitivity rather than a biological reality (26) The discrepancy between CHI and HAI might also be due to Norwegian TBE variants This is contradicted by the close antigenic interrelationships shown in the TBE virus complex (8) and also by the close relationship to a Central European TBE virus of the Norwegian strains isolated so far (28) The clinical histories of patients with »high« antibody titres may indicate that TBE virus plays a role in human pathology in Norway Further studies on selected groups of patients may clarify this aspect

The demonstration in both human and bovine sera (24, 27) of antibodies to two tick borne virus points to mixed infections We have shown experimentally that Uukuniemi virus may modify the lethal TBE virus infection in mice that mixed infections in some combinations may aggravate the effects on the host and that chronic CNS disease may develop in some instances (29)

Serological screening of the same human and bovine sera indicates that TBE virus is more active in *I ricinus* invaded areas than Uukuniemi and Tribec virus (25 and unpublished results)

The serum panel and the clinical information on which this work is based would not have been available without the kind contributions of Professor *Gunnar Haukenes* and his staff at the Department of Microbiology The Gade Institute University of Bergen I am very grateful to them

Einar Brunvold has contributed expert technical assistance

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PSEUDOMONAS CEPACIA BACTERÆMIA DUE TO INTRINSIC CONTAMINATION OF AN ANÆSTHETIC

Bacteriological and Serological Observations

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K SIBONI and P SØGAARD[2]

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Borghans J G A Hosli Marja Th C Olsen H Ravn Elsebeth M Siboni K & Søgaard P *Pseudomonas cepacia* bacteræmia due to intrinsic contamination of an anæsthetic Bacteriological and serological observations Acta path microbiol scand Sect. B 87 15–20 1979

In November December 1977 an epidemic of bacteræmia due to *P cepacia* was observed in Odense Denmark (nine patients) and in Nijmegen Holland (seven patients) All patients recovered The epidemic was [illegible] from the patie[illegible] patterns and [illegible] in two passage[illegible] p hydroxi benzoate 0 5 mg/ml which promoted the growth of the microorganism inocula of 2–20 cfu were sufficient to initiate growth in the drug or preservative These facts indicate the inadvisability of using p hydroxi benzoates as preservatives in vials The strain was inhibited at temperatures above 38 5°C corresponding to the recovery of the patients after a period with fever above 39°C Fourteen out of 15 patients examined had agglutinin titres ≥ 320 while 36 blood donors had titres < 40 Of 12 patients with postoperative fever in the same period whose blood cultures did not yield *P cepacia* three had titres > 320

Key words Anæsthetic hospital infection intrinsic contamination *Pseudomonas cepacia* serology

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In November December 1977 an epidemic of bacteræmia due to *Pseudomonas cepacia* was observed in Odense Denmark (nine patients D1–D9) and in Nijmegen Holland (seven patients H1–H7) All patients recovered from the acute episode The epidemic was traced to intrinsic contamination of batches 77 E 13/607 and 77 F 07/606 of the anæsthetic fentanyl (Haldid®)

The epidemiology of the outbreak and the clinical condition of the patients has been described by *Siboni et al* (1979) In the present paper the results of bacteriological and serological investigations will be reported the growth of the isolates in media with different content of carbon sources and preservatives and at different temperatures in order to elucidate the background of the epidemic and to give a quantitative basis for the febrile and serological response in the patients

MATERIALS AND METHODS

Strains used in bacteriological experiments

P cepacia

D 60 541 isolated Nov 23 1977 from the blood of patient D 1

D 61 354 isolated Dec 4 from the blood of patient D 3

TABLE 1 *Growth of Epidemic Strains of P cepacia in Hutner s Medium without and with Different Carbon Sources*

Isolate no	60 541	61 354	62 622	62 669	
Medium					
Hutner s medium without carbon source	6 1	6 3	6 2	6 3	t = 9 83 f 3 P < 0 01
Hutner s medium with methyl p hydroxi benzoate 0 5 mg/ml	7 0	7 1	7 0	6 8	
Hutner s medium with arginine 2 mg/ml	7 6	7 6	7 6	7 5	

(log cfu/0 1 ml after three days growth in 2nd passage)

The isolates grew well in ammonium citrate and malonate and in two passages in Hutner s minimal medium containing glucose, 1 mg/ml arginine 2 mg/ml methyl p-hydroxi benzoate 0 5 mg/ml and β hydroxi butyric acid 5 mg/ml and formed inclusions in the last named medium

Identical isolates were obtained from 15/99 vials of batch E (10 1–10 1 5/0 1 ml) and from 44/46 vials of batch F (10 4–10 5/0 1 ml) Among routine antimicrobials the isolates were sensitive only to cotrimoxazole and to nalidixic acid This is in accordance with the sensitivity pattern described by Woods & Young (1975) for the majority of P cepacia strains

Growth in the Preservatives and in Fentanyl

The epidemic isolates grew in two passages in Hutner s medium containing methyl p hydroxi benzoate, 0 5 mg/ml while the AB stock strains did not None of the strains could grow in the second passage at 1 mg/ml Table 1 shows the titres three days after the second passage in Hutner s medium with different carbon sources The preservative as well as arginine promoted the growth of the four isolates

Table 2 shows the results of a growth experiment in the drug and in pure p-hydroxi benzoate solution Inocula of 2–20 cfu were sufficient to initiate growth The final titres were not appreciably higher

TABLE 2 *Growth of* Pseudomonas cepacia *Isolate 60 541 in Fentanyl at Different Temperatures and in the p hydroxi benzoate Preservative*

	log inoculum in 5 ml	day 1	2	3	4	7	14	generation time (h)	on day
methyl p hydroxi benzoate 0 5 mg/ml + propyl p-hydroxi benzoate 0 05 mg/ml at room temperature 22–24°C	0 3	<0	1 2	2 9	5 3	5 3	5 5	3 6	2–4
fentanyl room temperature	0 3	<0	0 6	2 9	5 0	5 0	4 3	3 3	2–4
fentanyl 30°C	1 3	0 3	2 7			6 1	5 7	3 0	1–2

(log cfu per 0 1 ml)

D 62 622 isolated form a 10 ml vial containing fentanyl batch 77 E 13/607

D 62 669 isolated from a 10 ml vial containing fentanyl batch 77 F 07/606

AB 1164 received from Dr *H Frederiksen* Department of Diagnostic Bacteriology Statens Seruminstitut Copenhagen

AB 1424 received from Dr *H Frederiksen* Department of Diagnostic Bacteriology Statens Seruminstitut Copenhagen

AB 1425 received from Dr *H Frederiksen* Department of Diagnostic Bacteriology Statens Seruminstitut Copenhagen and described by *Bremmelgaard* (1975)

Strains used in serological experiments

P cepacia

B 1130 isolated Dec 15 1977 from the blood of patient H 1

B 1132 isolated Dec 16 from the blood of patient H 1

B 1134 isolated Dec 17 from the blood of patient H 7

B 1135 isolated Dec 17 from the blood of patient H 6

D 60 541 isolated Nov 23 from the blood of patient D 1

P 8737/1 isolated from 10 ml vial no 1 containing fentanyl batch 77 F 07/606 (big colony variant)

P 8737/2 isolated from the same vial (small colony variant)

P 8737/5 isolated from 10 ml vial no 5 containing fentanyl batch 77 F 07/606

P aeruginosa serotype 6 (Habs) received from Dr *T H Siem* Gemeenteziekenhuis Arnhem (*Siem* 1972)

P maltophilia received from Dr *J Borst* Afdeling Bijzondere Bakteriele Determinaties Rijksinstituut voor de Volksgezondheid Bilthoven Holland

Serum samples

Group 1 15 samples taken 7–51 days after injection of fentanyl from patients with *P cepacia* positive blood cultures

Group 2 12 samples taken 12–47 days after injection of fentanyl from patients with *P cepacia* negative blood cultures

Group 3 9 samples taken 12–41 days after injection of fentanyl from patients without postoperative fever

Group 4 36 samples from blood donors

Bacteriological Methods

The isolation of the strains from patients and fentanyl vials has been described by *Siboni et al* (1979)

The ability of the isolates to grow on glucose β hydroxi butyric acid arginine and methyl p hydroxi benzoate was tried in Hutners minimal medium (*Holding & Collee* 1971) The first passage was made from an overnight culture in Hutner s medium without carbon source

O/F media with carbohydrates were as reported by *Hugh & Leifson* (1953) Other media and staining of flagella were as used by *Siboni* (1976) Sensitivity tests were performed with Sensitabs® (Rosco DK 2630 Taastrup) or with Multodiscs® (Oxoid Basingstoke U K) *Growth rate at different temperatures* was tested as described by *Olsen* (1966)

The Drug Fentanyl (Haldid®) and the Preservatives

The anæsthetic fentanyli citras 78 5 µg/ml was dispensed by the manufacturer in 10 ml vials To the solution had been added the preservatives methyl p hydroxi benzoate 0 5 mg/ml and propyl p hydroxi benzoate 0 05 mg/ml corresponding to a total of 0 049 g/100 ml of p hydroxi benzoic acid The vials had not been sterilized after closure

Batch 77 E 13/607 (batch E) had been produced in May 1977 batch 77 F 07/606 (batch F) in June 1977 The two batches had been used in Danish hospitals from about August 31 and they were withdrawn Dec 14 and Dec 9 respectively In Holland batch F was withdrawn Dec 19 1977 Batch E had not been sold to Holland For further epidemiological details see *Siboni et al* (1979)

Growth in the drug or preservative was quantified by using bacteria from an overnight culture twice washed in sterile distilled water After control for sterility vials containing the drug were inoculated with volumes of 0 1 ml from a dilution series 10^1–10^7 of the washed bacteria Only the vial showing growth with the smallest inoculum was examined further The same experiment was repeated with sterile distilled water containing only the preservatives

Serological Methods

Production of O antigens Suspensions of cultures were adjusted to McFarland standard no 3 (*Pak & Suggs* 1974) and boiled for 1 hour After cooling they were stained by addition of crystalviolet (final concentration 0 1 mg/ml)

Microtiter agglutination tests Serum samples were examined before and after inactivation for ½ h at 56°C in two fold dilution series (1 10–1 1280) using 0 025 ml serum dilution and the same volume of antigen in each cup

Cross absorption tests (modified from *Cruickshank et al* 1975) were carried out on serum samples from patients D 4 and H 1 with *P cepacia* isolates Nijmegen B 1132 P 8737/5 and Odense 60 541 and with the *P maltophilia* stock culture

RESULTS

Isolation Identification Sensitivity Pattern

Blood cultures from nine Danish and seven Dutch patients yielded identical aerobic non fluorescent lophotrichous Gram negative rods with the characters described by *Siboni et al* 1979) the strain belongs to *Pseudomonas cepacia* (*Doudoroff & Palleroni* 1974) It differs from typical strains in not reducing nitrate to nitrite but accords in this respect with a minority of the strains described by *Bremmelgaard* (1975) and *Snell et al* (1972)

TABLE 5 *Antibody Titres against P cepacia Antigens (Inactivated Serum Samples)*

Titer	Group 1 Patients with *P cepacia* positive blood cultures	2 Patients with *P cepacia* negative blood cultures	3 Patients without postoperative fever	4 Blood donors
<20	0	3	3	16
20	0	2	2	11
40	0	3	2	9
80	1	1	2	0
160	0	0	0	0
320	3	0	0	0
640	3	0	0	0
≥1280	8	3	0	0
Totals	15	12	9	36

None of the serum samples agglutinated *P aeruginosa* H 6
All serum samples from groups 2 and 4 were negative against *P maltophilia* Six samples from group 1 and one from group 3 had titres ≤ 40 against *P maltoph l a*

The stock strain AB 1425 differed from the epidemic isolates in not growing at 4°C and in showing a considerable growth inhibition already at 38°C (Table 3)

Based on the results given in Table 3 the optimal growth temperature for all strains were 30 and 35°C

Serology

Agglutination tests More than two-fold differences in titres of one serum against antigens from different *P cepacia* isolates *in the same run* were not seen Likewise we did not find more than four fold differences in titres of different runs Generally titres of serum samples after inactivation were equal to or higher than titres before inactivation

All but one patient with *P cepacia* positive blood culture and three patients with *P cepacia* negative blood cultures had high titres against the eight *P cepacia* antigens Table 5 *illustrates the differences* between titres obtained in the four groups of samples investigated

Crossabsorption tests The Danish isolate 60 541 and the Dutch isolates B 1132 and P 8737/5 eliminated all agglutinins from the Danish and Dutch serum samples

The addition of the *P maltophilia* suspension did not affect the agglutination titres of the serum samples

DISCUSSION

The biochemical characters of the epidemic strain permitted the diagnosis of *Pseudomonas cepacia* (*Siboni et al* 1979) which is a somewhat variable taxon (*Bremmelgaard* 1975 Doudoroff & Palleroni 1974 *Snell et al* 1974)

In the unbroken vials the titres about $10^{4.5}$/0 1 ml were no higher than those obtained by other investigators with *P cepacia* in distilled water (*Carson et al* 1973) Under aerobic conditions – in open vials (Table 2) – the final titres were about the same In Hutner s saltsufficient *medium without* carbon source the titres increased with about 1 $\log_{10}$ (Table 1) and the preservative methyl p hydroxi benzoate as well as arginine could serve as a carbon source in the concentration used 0 5 mg/ml while 1 mg/ml was inhibitory to growth As the stock strains (*P cepacia* AB numbers) did not grow at either concentration these findings suggest the selection of a resistant clone this as well as the small inoculum sufficient to initiate growth (Table 2) indicates the inadvisability of using p hydroxi benzoates as preservatives in vials

The suggested development of resistance to p hydroxi benzoates agrees with the findings of [illegible]

[illegible] of vials was 3 0–3 6 hours (Table 2) but may have been much longer in unbroken vials as is the case with *P aeruginisa* in unvented vacuum blood culture bottles (*Braunstein & Tomasulo* 1976 *Knepper & Anthony* 1973)

The inhibition of growth of the strain at temperatures above 38°5 C may explain the recovery of the patients after a period with fever

TABLE 3 *Maximum Titres of Five* Pseudomonas cepacia *Strains at Different Temperatures*

Temperature		60,541	61,354	62,622	62,669	AB 1425
4°C	log maximum titres	6 5	7 5	8 0	7 5	NG
	number of days *until maximum titres*	21	17	20	20	
10	log maximum titres	7 0	8 5	6 0	7 5	7 0
		14	17	20	18	37
20	log maximum titres	7 0	8 5	7 5	8 0	8 0
		4	4	4	4	4
30	log maximum titres	7 5	8 5	7 0	8 0	7 5
		2	2	3	2	2
35	log maximum titres	7 5	9 0	6 5	8 5	8 0
		2	2	2	2	2
37	log maximum titres	7 5	8 5	5 0	8 0	7 0
		3	3	5	2	3
38	log maximum titres	7 0	9 5	4 5	7 5	1 5
		4	4	4	3	4

NG no growth
For each temperature the figures in the second row state the number of days before the maximum titres were obtained

than those of the contaminated vials and no higher in fentanyl than in the pure preservatives The generation time was 3 0–3 6 hours

Growth Rate at Different Temperatures

The maximal titres obtained at each temperature and the time required to reach these titres are given in Tables 3 and 4

The clinical isolates 60,541 and 61,354 and the fentanyl isolates 62,622 and 62,669 reached about the same titres at temperatures between 4 and 38°C (Table 3)

At 38°5 C all but one isolate, 61,354, showed definitely lower titres, and at 39°C growth of all isolates was much reduced or completely inhibited (Table 4)

TABLE 4 *Maximum Titres of Five* Pseudomonas cepacia *Strains at Temperatures Close to the Upper Limit of Growth and at 35°C*

Temperature		60 541	61 354	62 622	62 669	AB 1425
35°C	log maximum titres	6 1	6 9	6 3	7 7	7 7
	number of days until maximum titres	3	2	2	3	2
38 5	log maximum titres	3 2	7 1	3 2	2 7	NG
		8	5	7	7	
39	log maximum titres	NG	3 7	NG	1 1	NG
			6		4	
39 5	log maximum titres	NG	1 7	NG	NG	NG
			5			

NG no growth
For each temperature the figures in the second row state the number of days before the maximum titres were obtained

ELECTRON MICROSCOPY AND IMMUNOPEROXIDASE STAINING OF *STREPTOCOCCUS MUTANS* DURING CONTROLLED GROWTH IN TWO DIFFERENT MEDIA

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Berthold C H Berthold P & Nord C E Electron microscopy and immunoperoxidase staining of *Streptococcus mutans* during controlled growth in two different media Acta path microbiol scand Sect B 87 21–28 1979

Streptococcus mutans subspecies *sobrinus* serotype *d* (B13) was cultivated *in a fermentor* under controlled conditions in two different media a complex proteose peptone medium and a defined minimal medium (C4) Specimens from different growth phases were examined by electron microscopy and tested electron immunohistochemically Irrespective of the growth medium used no differences were observed in the immunohistochemical staining pattern of bacteria during the lag the exponential and the early stationary phases Specimens obtained several hours after exponential growth contained areas where the bacteria showed staining that ranged from a strong deposit of reaction product to no deposit This appearance seems partly to explain the differences in the intensity of immunohistochemical staining of certain bacteria observed in dental plaques stained for identification of *S mutans* subspecies *sobrinus*

Key words *Streptococcus mutans* glycol methacrylate electron microscopy immunohistochemistry and horseradish peroxidase

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The investigation of oral microorganisms by means of immunofluorescence and by electron microscopy has recently attracted considerable interest (*Bleiweis* 1976) However ordinary electron microscopy is insufficient for the identification of for instance different oral viridans streptococci some of which are considered cariogenic (*Fitzgerald* 1976 *Holt & Leadbetter* 1976) Attempts have therefore been made to identify *in situ* growing dental plaque microorganisms (*S mutans* and *S sanguis*) by the use of electron immunohistochemistry (*Lai et al* 1974 *Berthold* 1975) In the case of *S mutans* it was found (*Berthold & Berthold* 1978) that immunoperoxidase staining of ultrathin sections of two-day-old dental plaques gave rise to a distinct electron-dense reaction product which formed a characteristic thick continuous layer on the walls of some bacteria This staining pattern, type A was similar to that noted on sectionstained bacteria of [illegible]

[illegible] and the [illegible]

[illegible] combination of these factors The present investigations was therefore carried out with [illegible]

above 39°2 C Also the Danish stock strain was inhibited at 38°C (Table 3) As the strain examined by *Carson et al* (1975) and 13 of the 35 strains examined by *Snell et al* (1972) were able to grow at temperatures above 44°C, the upper limit of temperature tolerance may vary in different parts of the world as is the case with *Flavobacterium meningosepticum* (*Olsen* 1966)

The serological results make it highly probable that at least three patients with postoperative fever but without recognized bacteræmia belonged to the epidemic (Table 5, group 2) The postoperative clinical features in these patients were similar to those of the patients in whom bacteræmia had been demonstrated (*Siboni et al* 1979) This result demonstrates the usefulness of serological methods in elucidating the true extent of hospital epidemics due to *P cepacia*

The cross absorption test showed that the Danish and Dutch strains had identical antigens, this result as well as the identity of biotypes and sensitivity patterns supports our assumption that the two outbreaks had a common origin

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Abbreviations used in micrographs and legends Cpl = »cotton pellet like« bodies Cw = cell wall Cwf = cell wall fragment EM = electron micrograph H rp = horseradish peroxidase reaction product M = mesosome N = nuclear region and Or = outer rim of cell wall Asterisks indicate notches in the H rp localized at the site of a cross wall formation

All EMs show glutaraldehyde fixed bacteria belonging to *S mutans* serotype *d* strain B 13 All sections were contrasted with uranyl acetate and lead citrate Unless otherwise indicated the bar in the upper part of each figure indicates 0 2 µm

Fig 1 Exponential phase Proteose peptone medium Vestopal embedding The bacteria appear to be well preserved and contain numerous ribosomes The cell wall consists of two parts an inner highly electron dense layer and an outer thicker less electron-opaque layer with an outer rim of increased electron density and a fuzzy coat Closely apposed bacteria show tiny fibrillar interconnections (arrow) The dividing bacteria disclose cell wall bands (encircled) × 50 000

Fig 2 Same sample as shown in Fig 1 GMA embedding The appearance of the bacteria is similar to that in Fig 1 Note the GMA-dependent patchwise loss of connection between the cytoplasm and the cell wall and the empty looking defects in the cytoplasm The cytoplasm contains numerous ribosomes × 50 000

Fig 3 Exponential phase Proteose peptone medium GMA-embedding The specimen was immunoperoxidase section stained using anti *S mutans sobrinus* serum All bacteria are surrounded by a characteristic highly electron dense thick coat of H rp which at some sites show notches (asterisks) Note »unstained« cross walls (arrow) × 20 000

Fig 4 Same specimen as in Fig 3 The specimen was immunoperoxidase section stained using normal rabbit serum Note the complete lack of H rp Compare with Fig 3 × 20 000

Fig 5 Stationary phase (16 hours after exponential growth) Proteose peptone medium Vestopal embedding The specimen contains both »normal looking« bacteria (arrow compare with Fig 1) more or less d[illegible]

[illegible] The cytoplasm contains dense bodies of many different shapes The highly electron-dense character of the inner layer of the cell wall found in the »normal looking« bacteria is more or less lost in the disorganized ones (double arrow) × 50 000

Fig 6 Same sample as in Fig 5 GMA-embedding The specimen was immunoperoxidase section stained using anti *S mutans sobrinus serum* Several bacteria are surrounded by a thick highly electron-dense H rp whereas others disclose a patchwise distribution of the H rp Some bacteria with a morphology similar to fully stained members of the culture lacked H rp (indicated by white rings) × 20 000

Inset – from the same section as in Fig 6 – showing cell wall fragments on which a heavy H rp has developed × 16 000

MATERIAL AND METHODS

Bacterial Strain

S mutans serotype *d* strain (B 13) isolated from human dental plaque by *Edwardsson* (1968)

Growth Media

Two growth media were used a complex proteose peptone medium (*Linder & Nord* 1971) supplemented with 1% glucose and a chemically-defined minimal medium (C 4) specially developed for growth of *S mutans* (*Carlsson* 1970 1971)

Cultivation Technique

Stirred fermentors with working volumes of 1 0 l were used (Biotec FL 101 Biotec Stockholm Sweden)

[illegible]

cultures in each medium were studied The C 4 medium cultures were grown under anaerobic conditions The cultivations were run for 24–70 hours and 6–12 samples were taken from each culture

Preparation of Inocula

The inocula were prepared from overnight cultures grown under static conditions in bottles containing proteose peptone medium The bacteria were washed once in phosphate buffered saline (PBS lacking Mg^{++} and Ca^{++} pH 7 2) resuspended in fresh medium appropriate to the experiment and then added to the fermentor to an initial dry weight of 0 2 mg/ml

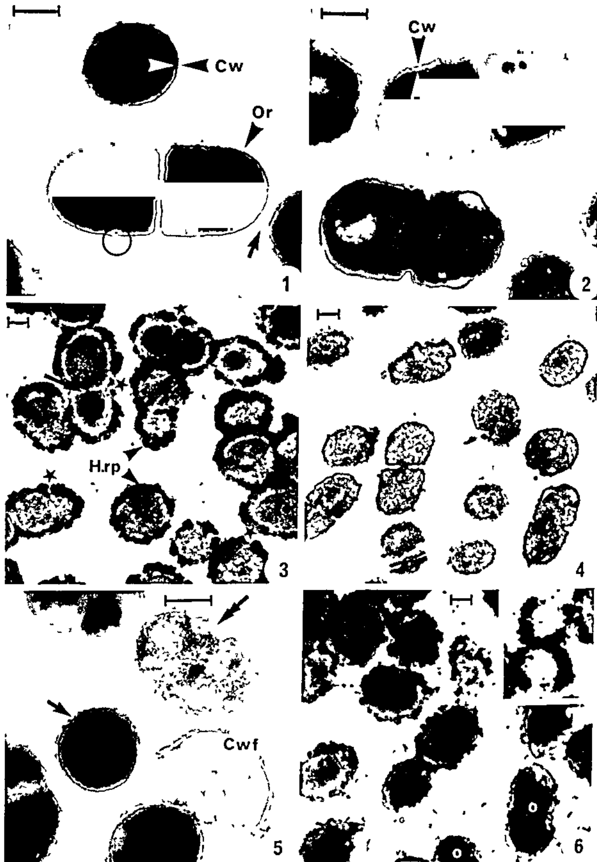
Determination of Bacterial Growth

A 10 ml amount was taken from the cultures at intervals centrifuged at 4 000 × *g* for 15 *min* at 4° C washed once in 0 01 M phosphate buffer pH 7 0 and dried at 110° C for 18 h before weighing All weights were corrected for the weight of the buffer salts

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Samples were taken at the same intervals as for determination of bacterial growth (cf *Berthold & Berthold* 1978) The treatment of the bacterial specimens differed according to the embedding medium used Vestopal W (*Ryter & Kellenberger* 1958) or glycol methacrylate (GMA *Rosenberg et al* 1960 *Leduc & Bernhard* 1967)

Vestopal embedding The glutaraldehyde used for fixation was prepared from a 25% stock solution


Cw
Or
1
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2
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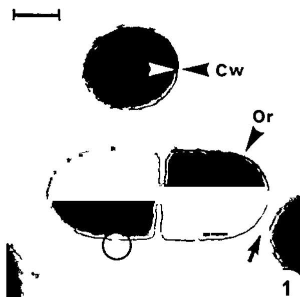
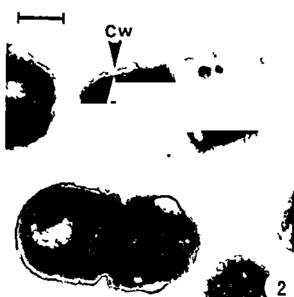
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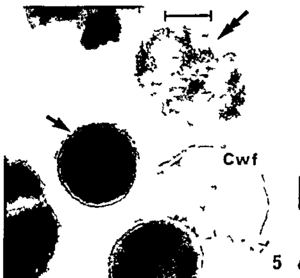
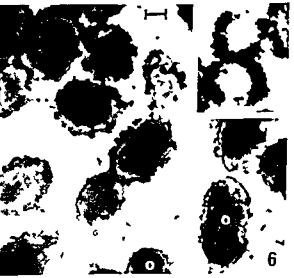
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Vestopal embedding The glutaraldehyde used for fixation was prepared from a 25% stock solution

purified by shaking with active carbon (*Fahimi & Drochmans* 1965) and dissolved in a phosphate buffer (*Karlsson & Schultz* 1965) with an osmolarity of 300 mOsM and pH of 7 2–7 4 to a final concentration of 2 5% The specimens were fixed for 4 hours rinsed in the buffer overnight and postfixed in 2% OsO_4 in veronal acetate buffer 300 mOsM pH 7 2–7 4 for 1 hour All procedures were carried out at 4° C Dehydration was performed in a graded series of acetone with a subsequent infiltration of Vestopal W and polymerization at 45° C for 24 hours and at 60° C for 48 hours

GMA embedding The specimens were fixed in 2 5% glutaraldehyde dissolved in phosphate buffer (see above) for 1 hour at 4° C rinsed in the buffer overnight stepwise infiltration with GMA and polymerized at 4° C for 24 hours under ultraviolet light (cf *Berthold & Berthold* 1978)

Sectioning Sectioning was performed on an LKB 4800 Ultrotome (LKB Bromma Sweden) equipped with a glass knife Sections from the Vestopal specimens were picked up on one hole formvar carbon coated copper grids and contrasted with both uranyl acetate and lead citrate (*Brody* 1959 and *Reynolds* 1963) Sections from the GMA specimens were picked up on one hole formvar carbon coated platinum grids and submitted to immunohistochemistry Some sections were osmified with 2% OsO_4 in veronal acetate buffer and double contrasted with uranyl and lead (*Brody* 1959 and *Reynolds* 1963)

Immunohistochemical staining The GMA sections were stained according to a previously described indirect immunoperoxidase method using a defined anti *S mutans sobrinus* γ globulin for the first incubation (*Berthold & Berthold* 1978) The specimens were examined in a Philips EM 300 or 301 operated at 80 kV

Controls

GMA sections incubated in PBS only or submitted to a first incubation with normal rabbit γ globulin (cf *Berthold & Berthold* 1978) were used as controls

Chemicals

All chemicals were of analytical grade unless otherwise stated Proteose peptone growth medium was obtained from Difco (Detroit Mich USA) The constituents for the buffers acetone 3 3 diaminobenzidine tetrahydrochloride and uranyl acetate were obtained from Merck (Darmstadt Germany) The lead nitrate and sodium citrate were obtained from Hopkin & Williams (Chadwell Heath England) Stock solutions of glutaraldehyde were obtained from Union Carbide Co (Chicago Ill USA) The osmium tetroxide was obtained from Inra Automation AB (Stockholm Sweden) Vestopal W was obtained from Mme Martin Jaeger (Zurich Switzerland) and GMA from Polyscience Inc (Warrington Pa USA) Normal sheep serum sheep antirabbit IgG and normal rabbit γ globulin were obtained from the National Bacteriological Laboratory (Solna Sweden) and horseradish peroxidase (type VI) and lysine from Sigma Chemical Corp (St Louis Mo USA)

Fig 7 Exponential phase C 4 medium Vestopal embedding The bacteria are similar to those obtained from the proteose peptone medium except for the numerous cytoplasmic electron lucent spots »cotton pellet like« bodies Mesosomes are common × 80 000

Fig 8 Same sample as in Fig 7 GMA embedding Disruptions between the cell wall and the cytoplasm are common The morphology of the GMA-embedded bacteria are in all other aspects similar to the Vestopal embedded ones (cf Fig 7) × 80 000

Fig 9 Exponential phase C 4 medium GMA embedding The specimen was immunoperoxidase section stained using anti *S mutans sobrinus* serum All bacteria are surrounded by a thick highly electron dense coat of H rp Notches are indicated by asterisks Compare with Fig 3 × 20 000

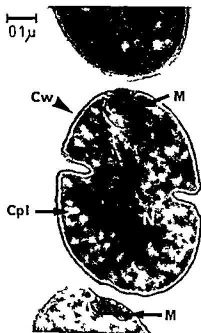
Fig 10 Stationary phase (24 hours after exponential growth) C 4 medium GMA embedding The specimen was immunoperoxidase section stained using anti *S mutans sobrinus* serum Several bacteria are covered with the highly characteristic electron dense coat of H rp whereas others more or less lack H rp (indicated by black circles) × 20 000

Fig 11 Stationary phase (16 hours after exponential growth) C 4 medium GMA embedding The specimen was immunoperoxidase section stained using anti *S mutans sobrinus* serum The picture shows part of a section where the immunoreaction was negative and where no H rp had developed Note the many bacteria with a disorganized appearance (cf Legend to Fig 5) × 7 500

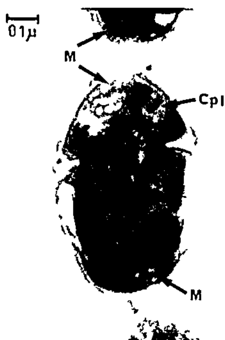
RESULTS

Cultivation in a Complex Proteose peptone Medium

Samples removed from the lag phase the exponential phase and the stationary phase (4 hours after exponential growth) The general electron microscopical appearance of Vestopal-embedded and GMA embedded samples was roughly similar (Figs 1 and 2) The bacterial cell wall was 25–30 nm thick It consisted of two layers of homogeneous appearance (1) an outer moderataly electrondense layer about 15 nm thick and covered by a serrated high-density rim 2–3 nm thick and (2) an inner highly electron dense layer 7–10 nm thick Some bacteria disclosed wall bands (cf *Higgins & Shockman* 1971) The bacterial cytoplasma was separated from the surrounding cell wall by an electron lucent space 2–7 nm thick It was not possible to determine in the electron micrographs whether a plasma membrane divided the cytoplasm from the dense inner part of the cell wall (cf *Swanson et al* 1969) The cytoplasm contained mesosom



01μ
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M
Cpl
N
M
7



01μ
M
M
Cpl
M
8



H rp
9



H rp
10
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11

mes (Figs 1 and 2) and a central nuclear region (*Glauert* 1962) In the GMA embedded specimens the electron lucent space between cell wall and cytoplasm was unstable The cytoplasm of several bacteria seemed to have pulled away from the cell wall leaving irregularly shaped holes or »empty« spaces in between (Fig 2) The occurrence of these defects seemed to depend on the batch of GMA prepolymers used All samples contained many dividing cells as well as some disorganized ones

Irrespective of the growth phase immunoperoxidase section staining (GMA-embedded samples) gave the same highly characteristic picture (Fig 3) of bacteria surrounded by a thick highly electron dense layer (70–250 nm) of HPR reaction product (cf *Berthold & Berthold* 1978) which obscured the cell wall and its immediate surroundings The layer was notched or completely missing at those points where cross walls in different stages of development were seen Many complete cross walls appeared unstained whereas some carried a distinct layer of the reaction product

Samples removed 16 24 48 and 64 hours after exponential growth Although normal looking bacteria were observed in all samples the number of more or less disorganized bacteria and cell fragments seemed to increase with the age of the culture (Fig 5) Many bacteria had lost the high electron density of the inner cell wall layer and showed increased width of the subjacent empty space which in several cases was divided from the cytoplasm by a double contoured cell membrane like structure

Immunoperoxidase staining gave rise to a reaction product distributed on top of the bacterial cell wall the amount and distribution of which ran the whole gamut from a thick continuous layer over a comparatively thin and discontinuous rim to a few electron-dense specks (Fig 6) Many bacteria which were free of reaction product were also observed More or less intensively stained cell wall fragments were common (Fig 6 inset)

Cultivation in a Minimal Medium (Medium C 4)

Samples removed from the lag phase the exponential phase and the stationary phase (4 hours after exponential growth) The general electron microscopical appearance of the samples was roughly similar irrespective of the embedding medium (Figs 7 and 8) In contrast to bacteria grown in the proteose peptone medium those in the C 4 cultures showed a large number of diffusely demarcated globular cytoplasmic bodies of low electron density These bodies hereinafter designated »cotton pellet like« bodies were particularly distinct after Vestopal embedding

As judged qualitatively after immunohistochemical staining (Fig 9) neither the amount nor the distribution pattern of the HRP reaction product differed from that observed on bacteria cultivated in the proteose peptone medium

Samples removed 16 24 and 48 hours after exponential growth »Cotton pellet like« bodies were not seen and the samples were similar both as regards general ultrastructure and immunohistochemical staining (Fig 10) to those removed from the later stages of cultures grown on proteose peptone The samples from one of the cultures showed large areas where all microorganisms lacked reaction product (Fig 11)

Immunohistochemical Controls

No reaction product developed after incubation in control sera (Fig 4)

DISCUSSION

The present observations show that the electron microscopical appearance of *S mutans* serotype *d* (B 13) is in line with that given earlier for viridans streptococci in general and *S mutans* in particular (*Guggenheim & Schroeder* 1967 *Nalbandian et al* 1974 *Holt & Leadbetter* 1976) However in contrast to the distinct appearance of the bactarial plasma membrane after primary OsO_4 fixation (*Holt & Leadbetter* 1976) the plasma membrane of intact glutaraldehyde fixed *S mutans* serotype *d* (B 13) could not be identified This difference between OsO_4 and glutaraldehyde fixed bacteria has been discussed by *Swanson et al* (1969) These authors were unable to recognize the plasma membrane in glutaraldehyde fixed *Lancefield* group A streptococci and described the cell wall as consisting of an inner highly electron dense layer and an outer moderatly electron dense layer It has been suggested (*Swanson & Gotschlich* 1973) that this inner layer may be particularly rich in teichoic acid Conflicting with this is the opinion that teichoic acid is distributed throughout the cell wall (*Milward & Reaveley* 1974)

The occurrence of »cotton pellet like bodies in bacteria grown in the minimal medium constituted a clear morphological difference as compared with bacteria grown in the proteose peptone medium In view of earlier observations (*Cedergren & Holme* 1959 *de Persio et al* 1974) and the fact that *S mutans* growing under nutrient deficiency or in an acidic environment stores polysaccharides intracellularly (*van Houte & Saxton* 1971 *Mattingly et al* 1976 1977) it is likely that the »cotton pellet like« bodies represent storage granules which are consumed when the growth conditions deteriorate (*Gibbons & Socransky* 1962) These variations in the

general ultrastructural appearance of bacteria belonging to the same strain but grown under different conditions strongly support the view put forward by van Houte & Saxton (1971) and others regarding the impossibility of identifying *in situ* growing plaque microorganisms by morphological criteria alone

Immunohistochemical staining of bacteria removed from the cultures during the lag phase, the exponential phase and several hours after the end of the exponential phase showed bacteria with an HRP reaction product comparable in size and electron density to that described earlier as being useful for identification of plaque growing bacteria (*Berthold & Berthold 1978*) The cross walls of dividing cells were unstained whereas cocci found close together revealed cell walls completely covered with reaction product This suggest that the antigen does not become distributed in all parts of the cell wall until after the cross wall has been completed A notch like defect in the otherwise complete reaction product layer was constantly observed at the site of a cross wall formation Similar distinct notches in the HRP reaction product layer without a subjacent cross wall probably indicated the position of a future cross wall site (cf *Cole & Hahn* 1962) *Swanson et al (1969)* who studied the distribution of cell wall antigens in the *Lancefield* group A streptococcal cell wall using the immunoferritin labelling technique reported fewer ferritin particles in connection with a cross wall formation as compared with other parts of the wall

In samples removed many hours after the exponential growth the amount and distribution of the HRP reaction product varied considerably between the individual bacteria thus indicating a patchwise or even complete disappearance of antigenic sites The frequent occurrence in these samples of cell wall fragments many of which were covered with thick coats of reaction products is noteworthy In the dental plaque similar fragments would contaminate the matrix substance and might adhere to neighbouring bacteria Shedding of cell wall fragments from *S mutans* serotype *b* (Fa 1) has been reported by *Shockman et al* (1976) who pointed out the problems to which such pieces can give rise in the immunohistochemical localization and identification of *in situ* growing microorganisms

Although the present observations contradict the concept that cultivation of *S mutans* serotype *d* in a minimal medium affects the occurrence of cell wall antigens to an extent which is possible to detect qualitatively by the use of an immunohistochemical staining procedure they have shown that such an effect did take place during declination of the cultures Consequently, dental plaque microorganisms which after immunohistochemical staining for the identification of *S mutans* serotype *d* bacteria, show a moderate to-weak positive reaction described previously as a reaction product distribution pattern of type B (*Berthold & Berthold* 1978) may well represent *S mutans* serotype *d* bacteria in a state of declination

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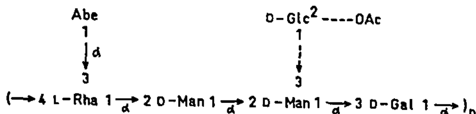


Fig 1 Structure of the O antigenic polysaccharide chain in the lipopolysaccharide from *S newport* serogroup C2, and *S kentucky*, serogroup C3, according to *Hellerqvist et al* (11)
In *S kentucky* a 2–O–acetyl–D–glucose residue is linked to D–galactose at the 4–position
Abbreviations used Abe, abequose, Glc glucose
Gal, galactose, Man mannose
Rha rhamnose
n = 4–16

and subsequent coupling to a protein should, at least theoretically, provide immunogens able to elicit monospecific antisera This was true also in practice when antisera against synthetic *Salmonella* O-antigens 2, 4 and 9 coupled to bovine serum albumin (BSA) were used for identification of *Salmonella* bacteria by indirect immunofluorescence (IFL) and by co-agglutination (COA) using sensitized protein A containing staphylococci (20–22) The high specificity of such antisera was also evident in passive haemagglutination quantitative precipitation, enzyme-linked immunosorbent assay (ELISA) and complement-mediated bactericidal tests (1, 5, 12)

The structure of the repeating unit of the O-antigenic polysaccharide chain in *Salmonella* serogroup C2 and C3 is shown in Fig 1 (11) The group-specific determinant of these serogroups is O antigen 8, which (analogous with O antigen 2 4 and 9) is probably represented by the disaccharide abequose $1 \xrightarrow{\alpha} 3$ rhamnose (11, 17 18) The disaccharide has been synthesized (6) and linked to bovine serum albumin (BSA) as an immunogenic carrier (via the synthesis of the phenylisothiocyanate disaccharide glycoside) (19) This paper presents the use of this synthetic disaccharide protein conjugate as immunogen in rabbits for production of specific O8 antiserum The serum was further tested for specificity in ELISA and used for identification of *Salmonella* serogroup C2 (O antigen 6 8) and C3 (O antigen 8, 20) bacteria by indirect immunofluorescence (IFL) and by co agglutination (COA) using sensitized protein A-containing staphylococci

MATERIALS AND METHODS

Bacterial strains The enterobacterial strains used in this investigation were obtained from the culture collection at the Department of Bacteriology National Bacteriological Laboratory Stockholm Sweden or from fresh faecal samples delivered to the laboratory for examination for pathogenic enteric bacteria The *Salmonella paratyphi* A strains came from Professor *L Le Minor* International Salmonella Centre, Pasteur Institute, Paris France Isolation and identification of the bacteria were carried out according to established methodology (4 13) *Salmonella* bacteria were isolated on desoxycholate citrate agar (Oxoid CM 35) and on brilliant green phenol red agar after enrichment in Rappaport s broth

Staphylococcus aureus strain Cowan 1 known to produce large amounts of protein A was used for the preparation of COA reagents The strain came from the collection of Professor *Tord Holme* Department of Bacteriology Karolinska Institutet Stockholm Sweden

Lipopolysaccharide extraction Lipopolysaccharides (LPS) from *Salmonella newport* (O antigen 6 8) *S thompson* (O antigen 6 7) and *S typhimurium* strain SH4809 (O antigen 4 5 12_2) were used for the enzyme-linked immunosorbent assay (ELISA) The LPS were extracted by phenol water from batch grown bacteria as described elsewhere (15)

Synthetic immunogen and immunization procedure Synthesis of the disaccharide *p*-nitrophenyl 3-O-(α abequopyranosyl)-α L rhamnopyranoside with antigen O8 specificity has been described previously (6) The hapten was linked to BSA via the synthesis of the phenylisothiocyanate disaccharide glycoside (19) The substitution of disaccharide was 8 mol per mol BSA as estimated by sugar and protein analyses described elsewhere (3 16) The disaccharide protein conjugate will be referred to in this investigation as AR BSA Immunization of rabbits with the synthetic immunogen was performed as described previously (20 21) Three New Zealand white rabbits weight 2 0–2 5 kg were immunized five times at intervals of one month and sera were drawn about ten days after each immunization *Salmonella* O factor serum 8 was prepared according to *Kauffmann* (13)

Enzyme-linked immunosorbent assay (ELISA) The ELISA was performed essentially as described earlier (2) Antisera against AR BSA were titrated and tested against BSA and LPS from *S newport* (O antigen 6 8) *S thompson* (O antigen 6 7) and *S typhimurium* strain SH4809 (O antigen 4 5 12_2) Sheep anti rabbit immunoglobulin was purified on insolubilized rabbit Ig and conjugated with alkaline phosphatase by the addition

of glutardialdehyde Conjugated material was separated from unconjugated enzyme and immunoglobulin by gel filtration on Sepharose 6B (Pharmacia AB Uppsala Sweden)

Immunofluorescence studies The indirect immunofluorescence method was used as described previously (20) The sheep anti rabbit immunoglobulin conjugated with fluoresceinisothiocyanate molar F/P ratio 4 6 was obtained from the Department of Immunology National Bacteriological Laboratory Stockholm Sweden The working titre of the conjugate was 1/40 A Leitz orthoplan fluorescence microscope with incident light and a mercury HBO–200 lamp as light source was used The fluorescence reaction was graded from 1 + to 4 + as described previously 4 + and 3 + indicating positive and 2 + and 1 + negative reactions (20)

Co agglutination studies (COA) The preparation of the staphylococci and their subsequent sensitization with anti AR BSA serum was performed as described previously (22) Briefly the procedure was as follows *Staphylococcus aureus* strain Cowan I was grown [illegible]

subsequently treated [illegible]

[illegible] heat treatment the washing procedure was repeated twice and the bacteria were finally resuspended to 10% as before 0 1 ml antiserum was added to 1 ml of the prepared staphylococci and allowed to react for three hours at room [illegible]

[illegible]

The performance of the COA test was as described previously (22) A few bacterial colonies were mixed with COA reagent on a glass slide which was rocked backwards and forwards for one minute Agglutination was registered as 2 + when clearly visible by the naked eye and as 1 + if a magnifying glass was needed for the observation

RESULTS

Enzyme linked Immunosorbent Assay (ELISA)

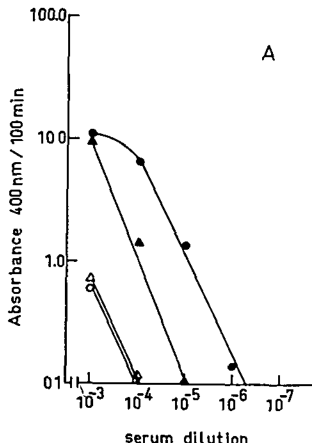
The specificity of the rabbit anti AR BSA serum used in the IFL and COA studies was tested in ELISA using BSA and LPS from *S newport* (O antigen 6 8) *S thompson* (O antigen 6 7) and *S typhimurium* strain SH4809 (O-antigen 4 5 12_2) as antigens Preimmunization sera were tested as well as sera drawn after three and five immunizations The end point titre defined as the reciprocal serum dilution value which gave an absorbance of 0 1 at 400 nm/100 min was 3×10^6 against *S newport* LPS and 10^5 against BSA with serum after three injections (Fig 2A) The titre against *S typhimurium* LPS was 10^4 Against *S thompson* LPS no titre above preimmunization level could be registered The corresponding values after five immunizations were 10^7 against *S newport* LPS 10^5 against BSA and 10^4 against *S typhimurium* LPS whereas no titre was registered against *S thompson* LPS (Fig 2B) The ELISA titres of conventional *Salmonella* factor O8 serum against the different antigens are shown in Fig 2C The titre was a hundred times lower against the homologous *S newport* LPS as compared to the titre of the anti AR BSA serum

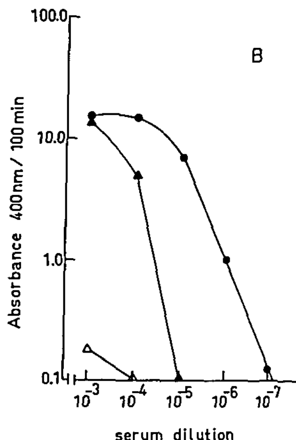
Indirect Immunofluorescence Studies Using Known Salmonella Strains

Antisera against AR BSA from three rabbits were titrated in twofold dilution steps and tested against a *S newport* indicator strain (O antigen 6 8) The end point titre defined as the last dilution which gave a 3 + reaction varied between 1/20 and 1/160 after three immunizations The corresponding values after five immunizations were 1/2560–1/10 240 When the antisera were tested against a *S agona* strain (O antigen 1 4 12) no fluorescence reaction could be seen at any dilution (serum tested in dilutions of 1/5 and upwards) Nor did preimmunization sera or anti BSA sera give any fluorescence reaction when tested with the same dilutions The last dilution which gave a 4 + reaction was chosen as the working dilution in the experiments reported hereinafter The working dilution was usually two or three twofold steps lower than the end point titre An antiserum obtained after three immunizations from the rabbit which gave the highest titre was used in the following experiments

The specificity of the anti AR BSA serum was tested against 174 known *Salmonella* strains representing the serogroups most usually *isolated* A (O antigen 1 2 12) B (O antigen 1 4 5 12) C [illegible]
12)
(9) [illegible] bacteria (most of the strains belonged to serogroup C2) gave a strong fluorescence reaction with the anti AR BSA serum (*Table 1*) Only in three instances was the staining reaction doubtful but when these strains were retested they all stained 4 + When 112 bacteria belonging to other serogroups were tested no fluorescence *reactions* occurred (Table 1) The investigation was performed without the investigators being aware of the strain or serotype used

When a conventional *factor O8* serum was used for the IFL investigation the 62 serogroup C2 or C3 bacteria also gave a strong fluorescence reaction





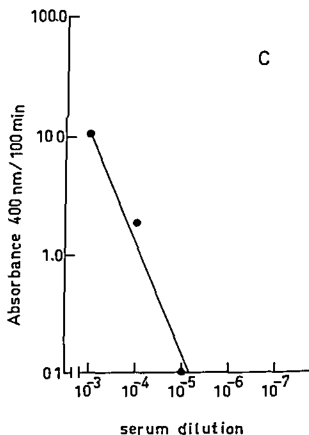


Fig 2 Titration of rabbit anti AR BSA serum and *Salmonella* factor O8 serum against BSA (coating dose 5 [illegible]

4 5 12_2 coating dose 1 µg/ml) All sera were tested diluted 10^{-3}–10^{-7} using tenfold dilution steps

A Anti AR BSA serum after three immunizations
B Anti AR BSA serum after five immunizations
C *Salmonella* factor O8 serum

Antigens ●——● *S newport*
○——○ *S thompson*
△——△ *S typhimurium*
▲——▲ BSA

However 1/12 strains belonging to serogroup C1 (O antigen 6 7) also stained (Table 1) The end point titre of the factor O8 serum tested against the indicator *S newport* strain (O antigen 6 8) was moreover *much lower viz 1/5 as compared to* that of the anti AR BSA serum It should be stressed however that the factor O8 serum used had been prepared for standard slide agglutination tests

TABLE 1 *Indirect Immunofluorescence (IFL) Studies of Salmonella Using Anti-AR-BSA and Factor O8 Sera*

Salmonella serogroup	No of strains	No positive in IFL[a]	
		AR BSA	Factor O8
A (O1 2 12)	25	0	0
B (O1 4 5 12)	25	0	0
C 1 (O6 7)	12	0	1
C 2–3 (O6 8 8 20)	62	62	62
D (O1 9 12)	25	0	0
E 1–4 (O3 10 3 15 3 34 1 3 19)	25	0	0
Total	174	62	63

[a] 4 + and 3 + reactions were scored as positive
Dilutions of antisera anti AR BSA 1/40 factor O8 1/5

TABLE 2 *Indirect Immunofluorescence Studies of Enterobacteria other than Salmonella Using Anti-AR-BSA serum*

Genus/species	No of strains	No positive in IFL[a]
E coli	236	0
Shigella	14	0
Citrobacter	5	0
Klebsiella	42	0
Enterobacter	16	0
Serratia	4	0
Proteus	67	0
Pseudomonas	42	0
Alcaligenes	4	0
Aeromonas	2	0
Acinetobacter	1	0
Yersinia enterocolitica	2	0
Yersinia pseudotuberculosis	7	0
Vibrio cholerae	5	0
Vibrio enteritidis	3	0
Vibrio parahaemolyticus	2	0
Bacteroides fragilis	50	1[b]
Total	502	1

[a] Scoring of the fluorescence reaction was as in Table 1
[b] Strain scored as negative when reexamined

Indirect Immunofluorescence Studies Using Known Non-Salmonella Enteric Bacteria

A further investigation was performed by testing 502 non *Salmonella* bacterial strains representing either other pathogenic enteric bacteria or the normal flora in the gut All strains belonging to *Enterobacteriaceae* as well as all strains of *Pseudomonas Alcaligenes Aeromonas Acinetobacter* and *Vibrio* were negative in the IFL test (Table 2) However, one strain of *Bacteroides fragilis* belonging to the subspecies *thetaiotaomicron* stained with the anti AR-BSA serum, but the reaction was not reproducible

TABLE 3 *Indirect Immunofluorescence Studies of Suspect Salmonella Bacterial Colonies*

Serogroup[a]	No of strains	No positive in IFL[b]
A (O1 2 12) 1 species	1	0
B (O1 4 5 12) 16 species	187	0
C1 (O6 7) 13 species	99	0
C2 (O6 8) 4 species	36	36
C3 (O8 20) 1 species	1	1
D1 (O1 9 12) 6 species	32	0
E1 (O3 10) 9 species	17	0
E2 (O3 15) 1 species	2	0
E4 (O1 3 19) 3 species	10	0
F (O11) 1 species	1	0
G1 (O1 13 22) 1 species	2	0
K (O18) 1 species	9	0
L (O21) 2 species	8	0
M (O28) 1 species	1	0
O (O35) 1 species	1	0
P (O38) 1 species	2	0
Non Salmonella	65	0
Total	474	37

[a] Serogroup classification is according to *Kauffmann* (13)
[b] Scoring of the fluorescence reaction was as in Table 1

TABLE 4 *Co agglutination (COA) Studies of Salmonella Bacteria Using Staphylococci Sensitized with Anti AR BSA Serum*

Salmonella serogroup	No of strains	No positive in COA
A (O1 2 12)	20	0
B (O1 4 5 12)	20	0
C1 (O6 7)	13	0
C2–3 (O6 8 8 20)	22	22[a]
D (O1 9 12)	21	0
E1–4 (O3 10 3 15 3 34 1 3 19)	19	0
Total	115	22

[a] Agglutination was clearly visible by the naked eye and appeared within seconds

Indirect Immunofluorescence Studies Using Unknown Enteric Bacteria from Faecal Samples

The anti AR BSA serum was subsequently used for detection of *Salmonella serogroup C2 or C3* bacteria isolated from faecal samples delivered to the laboratory for examination for pathogenic enteric bacteria Part of a suspect *Salmonella* colony was taken from either a desoxycholate citrate agar plate or a brilliant green phenol red agar plate and suspended in a drop of PBS on a glass slide for immunofluorescence investigation Part of the same colony was also inoculated in test tubes for standard biochemical reactions and on agar plates for subsequent serological investigation 474 suspect *Salmonella* colonies were tested Of these 37 stained with the anti AR BSA serum (Table 3) Conventional examination revealed that 36 of these strains belonged to *Salmonella* serogroup C2 and one to *Salmonella serogroup C3 both serogroups sharing* the O antigen 8 All 372 strains belonging to other *Salmonella* serogroups were negative as were 65 strains which were not *Salmonella* Among the non *Salmonella* strains *Proteus* species and *E coli* dominated

Co agglutination Studies

The applicability of the COA method introduced by Kronvall (14) for grouping of *Salmonella* serogroup C2 and C3 bacteria was proved by testing 115 known *Salmonella* bacteria representing serogroups A E All 22 strains belonging to *Salmonella serogroup C2 or C3* agglutinated strongly and within seconds with the anti AR BSA sensitized reagent (Table 4) No agglutination could be seen when 93 bacteria representing other serogroups were tested

An undiluted anti AR BSA serum was used to sensitize the staphylococci in these experiments

DISCUSSION

The use of synthetic *Salmonella* O antigen determinants as haptens coupled to an immunogenic carrier protein for production of group specific antisera has been shown previously to be a good alternative to conventional immunization with whole bacteria (20 21) In this study antiserum against the synthetic disaccharide abequose $1 \xrightarrow[\alpha]{} 3$ rhamnose representative of most likely O antigen 8 *coupled to BSA* was used for detection of *Salmonella* serogroup C2 and C3 bacteria by IFL and COA

The specificity was evident in IFL in that all 99 serogroup C2 and C3 bacteria among 1150 enteric bacteria tested stained brilliantly with the anti AR BSA serum (Table 1 3) No positive reactions occurred among 484 *Salmonella* bacteria belonging to other serogroups Among 567 non *Salmonella* enteric bacteria only one positive reaction could be seen i e one *Bacteroides fragilis ssp thetaiotaomicron* stained with the anti AR BSA serum (Table 2) The reason for this remains unknown and the reaction could not be reproduced

The anti AR BSA serum end point titres in IFL *varied between* 1/2560–1/10 240 after five immunizations Thus the anti AR BSA serum consumption is low and as a consequence highly economic The end point titre of a conventional *Salmonella* factor O8 serum was much lower viz 1/5 Moreover 1/12 *Salmonella* serogroup C1 strains (O antigen 6 7) gave a fluorescence reaction with the factor O8 serum (Table 1) The reason for this is probably the difficulty in absorbing the »O6 antibodies completely when preparing factor O8 *serum*

The specificity seen in IFL is a consequence of the specificity of the anti AR BSA serum ELISA titrations *revealed that the titre against the S newport* LPS antigen was approximately a thousand times higher than the titre against the *S typhimurium* LPS antigen (Fig 2 A B) Comparison with the *S typhimurium LPS* antigen confirms the specificity The only difference between the O8 and O4 antigens is that in the former abequose is

α1 3 linked to L rhamnose (which is 6 deoxy L mannose) whereas in the latter it is α1 3 linked to D-mannose The anti AR BSA titre in ELISA was also approximately a hundred times higher than the conventional factor O8 serum titre (Fig 2B C)

Also in the COA test the anti AR BSA serum was shown to be specific and useful All 22 *Salmonella* C2 and C3 bacteria tested agglutinated strongly and within seconds with the anti AR BSA sensitized reagent No positive reactions could be seen when 93 bacteria belonging to other *Salmonella* serogroups were tested (Table 4)

The high specificity of the anti AR BSA serum is probably a direct consequence of the »tailor made« immunogen which lacks the multitude of antigenic determinants involved when whole bacteria are used as immunogens Moreover disaccharide protein conjugates mostly give rise to IgG antibodies as compared to IgM antibodies elicited preferably with whole bacteria

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ELECTRON MICROSCOPICAL STUDY OF ANTIBODY BINDING TO *MYCOPLASMA GALLISEPTICUM* INDIRECT IMMUNOFERRITIN LABELLING

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Vinther O & Freundt E A Electron microscopical study of antibody binding to *Mycoplasma gallisepticum* Indirect immunoferritin labelling Acta path microbiol scand Sect B 87 37–44 1979

The ultrastructural aspects of the interaction of *Mycoplasma gallisepticum* with specific rabbit antibody have been studied In particular fixation conditions which allow the simultaneous preservation of cellular fine structure and membrane antigenicity have been established and applied in a procedure of indirect immunological labelling of the antibody-coated organisms with ferritin conjugated sheep anti rabbit IgG The advantages of working with agar embedded organisms in a multistep labelling procedure are discussed In membrane fractions of *M gallisepticum* prepared by osmolysis and freeze thawing only sealed membranes retained their antibody binding capacity Electron microscopical examination of break through colonies from immune growth inhibition zones revealed that the majority of cells in these colonies were destroyed sometimes limited only by a single layered membrane and without extracellular antibody coat An exception from this was the presumedly young cells in the periphery of colonies and in microcolonies which appeared to be intact and had a heavy antibody layer surrounding the cells Based on these characteristics a possible sequence of events is suggested eventually leading to destruction of mycoplasma organisms in immune growth inhibition zones

Key words *Mycoplasma gallisepticum* ultrastructure indirect immunoferritin labelling membrane antigenicity immune growth inhibition

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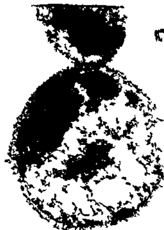
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The reactions of mycoplasma towards homologous antibodies differ in some respects from the reactions of other prokaryotes as demonstrated by the inhibition of growth on solid media of mycoplasmas exposed to specific antibodies (9) and by the inhibition of metabolism observed in liquid media (13) The possession of a triple layered cytoplasmic membrane as the only boundary of the mycoplasmas probably accounts in some way for their participation in these peculiar serological reactions

The mechanisms of the growth inhibition and metabolism inhibition reactions are largely unknown as are also the submicroscopical details of the interaction between antibodies and membrane delimited prokaryotes The main purpose of the present investigation therefore has been to determine conditions suitable for ultrastructural study of the interaction between antibodies and mycoplasma cells and membranes by an indirect immunoferritin labelling method with particular emphasis on fixation problems In addition some electron microscopical results are presented which relate directly to the growth inhibition test.

MATERIALS AND METHODS

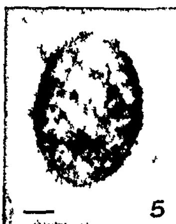
Organisms M gallisepticum (PG 31) was used throughout this investigation For use in immunoferritin labelling experiments the organism was grown on solid


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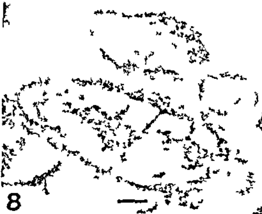

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Fig 1 *Mycoplasma gallisepticum* cell in agar grown colony treated with specific antibody after prefixation in 0 3% glutaraldehyde The cell shows a well preserved cytoplasmic content including one large cluster of ribosomes The exterior side of the cell membrane is covered with a uniform layer of antibody molecules about 15 nm thick Section post stained with magnesium uranyl acetate and lead citrate × 81000 The bar on this and all following micrographs represents 100 nm

Fig 2 Cell not treated with antibody An extramembraneous layer is not present but there are a few floccules of material adhering to the membrane Post stained with magnesium uranyl acetate and lead citrate × 81000

Fig 3 Cells incubated with specific antiserum and the ferritin label following prefixation with 0 3% glutaraldehyde Cells are structurally well preserved and are coated with a uniform layer of antibody decorated with apparently randomly scattered ferritin particles Section unstained × 81000

Fig 4 Experimental conditions as in Fig 3 except that prefixation was carried out with 1% glutaraldehyde Both large cells with little cytoplasm and smaller cells with dense cytoplasm are covered with antibody and ferritin One tiny cell also labelled is seen farthest to the right Section unstained × 63000

Fig 5 Cell treated with specific antibody and ferritin label after prefixation with 3% glutaraldehyde The cell structure is well preserved but a uniform antibody layer covering the cell membrane is not observed Instead a very thin layer and some floccules of material containing attached ferritin particles can be seen Section unstained × 67500

Fig 6 Cell incubated with specific antibody and ferritin label prior to any fixation The cell content seems to be shrunken whereas the membrane appears to be intact and covered with extramembraneous material and ferritin particles Ferritin particles in the interior of the cell are not observed Section unstained × 72000

Fig 7 Cell incubated with preimmune serum and ferritin label after prefixation with 0 3% glutaraldehyde A uniform extramembraneous layer is not present but some floccules of material containing attached ferritin particles can be seen close to the membrane Section unstained × 63000

Fig 8 [illegible]
ferritin [illegible]
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Fig 9 Unsealed membrane of *M gallisepticum* prepared by [illegible]

[illegible]
[illegible]ached Only a few ferritin particles are seen near the membrane Section stained with lead citrate × 90000

B medium (10) containing heat inactivated (56°C 45 min) horse serum Plates were incubated at 37°C for 3–4 days with an inoculum sufficient to give rather heavy growth consisting of a mixture of large colonies (~100–200 μm) and small colonies (<50 μm) For the production of immunization antigen and preparation of membrane fractions *M gallisepticum* was grown in aliquots of liquid B medium to yields of about 10^8 CFU/ml

Immunization procedure Rabbit hyperimmune serum against cells of *M gallisepticum* was produced essentially as described by *Black* (2) Blood samples were taken before immunization and five days after the last injection of antigen and the serum was stored at −20°C One batch of immune serum and of preimmune serum both heat inactivated and absorbed with the growth medium (3) before use were applied throughout The sera showed high titres and not detectable titres respectively when tested against the homologous antigen by the growth inhibition (2) and metabolism inhibition (19) methods

Indirect immunoferritin labelling Two layer immunoferritin labelling of mycoplasma cells in agar grown colonies was carried out both on unfixed colonies and on colonies prefixed *in situ* with varying concentrations of glutaraldehyde in veronal acetate buffer containing 0 01 M $CaCl_2$ pH 6 1 (VA buffer) (20) as described previously (22) The concentrations of glutaraldehyde tested were 0 3 1 and 3 per cent After fixation colonies to be examined were covered with one or two drops of warm (45°C) 1 per cent Noble agar (Difco) in VA buffer and after solidification small agar blocks (1 × 1 × 4 mm^3) generally containing one large colony and 3–4 small colonies were cut out The thickness of the agar overlay measured on sections in the electron microscope varied between 0 1 mm and 0 25 mm After a brief washing first in VA buffer then in 0 1 M tris buffer pH 7 4 the colony-containing blocks were incubated with rabbit anti mycoplasma serum diluted 1 40 in tris buffer for 1½ h at room temperature the blocks being agitated gently during the whole incubation time To remove unbound antibody the blocks were subsequently rinsed three times for 15 min by agitation in tris buffer Incubation with the second step reagent ferritin labelled goat anti rabbit IgG diluted in tris buffer took place for 2½ h at room temperature as described for incubation with the first step reagent Removal of unbound label was accomplished by a brief washing in tris buffer followed by overnight washing of the blocks in this buffer at 4°C and a final washing in VA buffer for 2 h all these steps being carried out by agitation Finally blocks were processed for electron microscopy as described below

The fer[illegible]

[illegible] The conjugate was purified by agarose gel chromatography (15) monitored by 310 nm

absorption and used in the dilution emerging from the column

Control experiments were carried out in which preimmune serum or tris buffer was substituted for rabbit immune serum in the first step using the same dilutions of preimmune serum as in the actual experiments

Membrane experiments A washed suspension of *M gallisepticum* cells in tris buffer containing about 10^9 cells was spun down at 27 000 *g* for 40 min and the resulting pellet resuspended in 5 ml distilled water Breaking of cells was accomplished by incubation of this suspension at 37°C for 30 min followed by ten cycles of alternate freezing at −70°C and thawing at room temperature After washing in 0 15 M NaCl membranes were collected by centrifugation at 37 000 *g* for 40 min and prefixed by resuspension in 0 3 per cent glutaraldehyde in VA buffer for one hour at room temperature The suspension of fixed membranes was subsequently distributed over the surface of a standard petri dish containing B medium agar The suspending buffer was allowed to drain into the agar and membranes on the agar surface were covered with a thin layer of melted agar After solidification small agar blocks were cut out and subjected to indirect immunoferritin labelling as described earlier The rabbit antiserum (against whole cells) was used in dilutions 1 100 or 1 200 and the conjugate in the dilution 1 60 Parallel control experiments were carried out in which preimmune serum or tris buffer was substituted for rabbit immune serum

Electron microscopy Agar blocks containing ferritin labelled mycoplasmas or mycoplasma membranes were fixed for one hour at room temperature in 3 per cent glutaraldehyde in VA buffer to improve preservation of fine structure and also to secure firm binding of the label during subsequent treatments These included fixation in 1 per cent OsO_4 fixative (14) for 4 hours followed by dehydration in acetone water mixtures and embedding in Vestopal W Sections were obtained with an LKB Ultrome III microtome and were examined either unstained or after section staining with lead citrate alone or lead citrate and magnesium uranyl acetate in a JEOL JEM 100B electron microscope

Growth inhibition experiments To study the ultrastructure of *M gallisepticum* cells in break through colonies within growth inhibition zones petri dishes containing solid B medium were equipped with a central well which was filled with 15 µl immune or preimmune serum diluted 1 10 Serum was allowed to diffuse radially for three days at 4°C after which the plates were inoculated evenly with 0 5 ml of a liquid culture of the organisms containing 5×10^7 CFU/ml After incubation for 4–5 days at 37°C growth inhibition zones with diameters of about 30 mm had developed on the immune serum plates whereas the preimmune plates showed uniform growth However a small number of colonies were observed within the growth inhibition zones and of these colonies not farther away from the central well than 5 mm were chosen for examination by electron microscopy using the preparation methods already described No attempts were made to label the cells with the immunological ferritin label or to remove unbound mycoplasma antibody

RESULTS

Indirect immunoferritin labelling Thin sectioned cells of *M gallisepticum* from agar grown colonies incubated with specific rabbit anti mycoplasma antiserum after prefixation with 0 3 per cent glutaraldehyde are shown in Fig 1 The ultrastructure of the cells was well preserved and the geometrical ribosome condensations characteristic of *M gallisepticum* (8) were evident as well as the »bleb« structures (17) on properly orientated cells In addition these cells were coated with an amorphous weakly contrasted 10–20 nm thick layer covering the cell membranes uniformly On untreated cells only an extremely thin membrane coat if any was observed together with a small amount of flocculent material adhering to the membranes (Fig 2)

Cells treated with specific antibody after prefixation with 0 3 per cent or 1 per cent glutaraldehyde and subsequently incubated with ferritin labelled goat anti rabbit IgG also showed a uniform extramembraneous layer about 15 nm thick besides the small electron dense particles identifying ferritin (Figs 3 and 4) In contrast cells incubated with specific antibody and the ferritin label after prefixation in 3 per cent glutaraldehyde consistently exhibited only a very thin layer with some floccules of material surrounding the cells (Fig 5) Some ferritin particles were also observed attached to this extramembraneous material

There was considerable difference in the preservation of fine structure of cells fixed in glutaraldehyde prior to the accomplishment of the immunological reactions and cells not prefixed The cytoplasmic content of prefixed cells was well preserved irrespective of the concentration of fixative used (Figs 3 4 and 5) whereas in unfixed cells the cytoplasm was shrunken and blebs were less pronounced (Fig 6) In both cases however membranes were intact as judged by the absence generally of outleaking intracellular material and of ferritin particles inside cells Unfixed or prefixed mycoplasma cells treated with preimmune serum and the ferritin label under the same conditions showed similar features with regard to preservation of structure as those already described but instead of a uniform antibody layer surrounding cells only patches of material adhering to the membranes were observed (Fig 7) Most of these patches contained attached ferritin

The distribution of the ferritin particles of the second step reagent on the sur

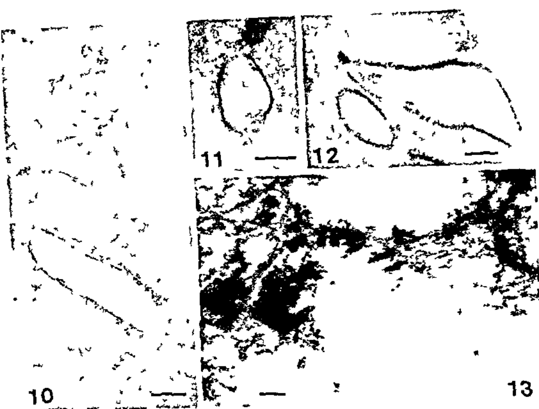


Fig 10 Sealed membranes from the same preparation as in Fig 9 The membranes are covered with a uniform layer of antibody decorated with ferritin particles Few ferritin particles can be seen in the cellular debris found in the preparation Section unstained × 90000

Fig 11 Membrane treated with preimmune serum and ferritin label A uniform extramembraneous layer is not present though some material containing ferritin can be seen near the membrane Section unstained × 108000

Fig 12 Membrane treated with tris buffer followed by ferritin labelling reagent No extramembraneous material or ferritin particles are present Section unstained × 81000

Fig 13 Cells found in »break through« colonies in immune growth inhibition zones Cells are nearly empty the remaining cytoplasm appearing in clumps The central cell shows remnants of geometrical ribosome structures and is limited on part of its circumference by an extremely thin single layered membrane Section stained with uranyl acetate and lead citrate × 67500

was irregular consisting of single particles or clusters or arrays of 2–4 particles apparently scattered at random around the circumference No special labelling of the bleb membrane region was observed (Fig 8) Ferritin particles were seen only rarely in the intercellular space Antibody layer and ferritin label were present on all the morphologically different forms of mycoplasma cells both on very small cells and on relatively large cells with little cytoplasm (Fig 4)

Membrane experiments When membranes of *M gallisepticum* prepared by osmotic lysis followed by repeated freeze thawing were incubated with antiserum towards whole cells and ferritin labelled goat anti rabbit IgG and examined in thin sections non sealed membranes or membrane fragments (Fig 9) were observed as well as structures resembling sealed membranes or empty cells (Fig 10) Both kinds of membranes contained adherent material though the layer of material was much more uniformly arranged in the case of sealed membranes In addition a relatively large number of ferritin particles was invariably found attached to the extramembraneous layer of the sealed membranes (Fig 10) whereas the non sealed membranes only occasionally showed a few attached particles (Fig 9). Ferritin labelling of the protein debris also found in the preparations was generally absent

When preimmune serum was substituted for immune serum sealed membranes showed only a patchy distribution of extracellular material labelled with ferritin (Fig 11) the unsealed membranes having the same appearance as that described for membranes incubated with immune serum In preparations with tris buffer substituted for rabbit serum extramembraneous material was absent as a rule and ferritin particles were not seen (Fig 12)

Growth inhibition experiments Electron micro scopical examination of › break through colonies found in a small number in zones of immune growth inhibition showed colonies populated to a high degree with deteriorated cells characterized by the presence of only a small amount of cytoplasm which appeared to be clumped or by the absence of cytoplasm At least on part of their surface a considerable number of these deteriorated cells were limited by an extremely thin single layered someti mes broken envelope or membrane (Fig 13) A uniform antibody layer was not observed on such cells At the periphery of such colonies and also in microcolonies made up of no more than 20-50 mycoplasma cells there were well preserved orga nisms containing ribosomes nuclear strands and intact membranes (Fig 14) These cells were surrounded by a thick layer of antibody as were also some empty cells located in the same areas Mycoplasmas in colonies grown on agar plates containing preimmune serum instead of immune serum did not show any of the above mentioned characteristics

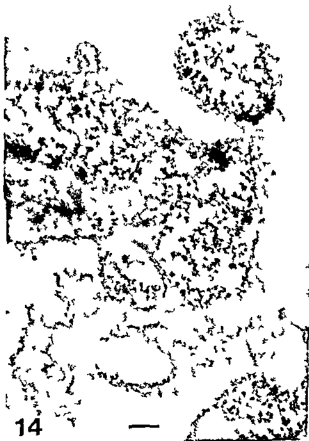


Fig 14 Cells from a microcolony found in immune growth inh b t on zone Both empty and intact cells filled with ribosomes can be seen Both kinds of cells are heavily coated with antibody Sect on stained w th uranyl acetate and lead citrate × 67500

DISCUSSION

Indirect immunoferritin labelling It has been shown in the present study that incubation of *M gallisepticum* cells with specific antiserum results in the attachment of a uniform layer of antibody molecu les to the cytoplasmic membrane of the organisms a layer which is visible in electron microscopical thin sections without further labelling A somewhat similar coat of externally adhering material resul ting from incubation of *A laidlawii* cells with specific antiserum in an investigation of comple ment induced lysis was presumed to be antibody but no demonstration of the immunoglobulin nature of the layer was given (5) On the other hand directly visible individual immunoglobul n molecu les radiating from the surface of virus particles have been demonstrated by the negative staining technique (1) The average thickness of the antibody coat measured in the present investigation about 15 nm is of the same order of magnitude as the overall linear dimensions of single IgG or IgM molecules (11) thus suggesting that the observed antibody coat on the mycoplasma membranes may be made up of a single layer of immunoglobulin molecules Signifi cant variations in the thickness of the coat as a function of serum dilution in the range 1 40 to 1 200 were not observed

The capacity of mycoplasma cells to bind specific antibody and the ferritin label in relation to the extent of prefixation of the organisms was investigated in order to establish optimal conditions for the simultaneous preservation of cellular fine structure and membrane antigenicity Prefixat on with glutaraldehyde in the range of 0 3 to 1 per cent was found to fulfil both these requirements Lack of prefixation resulted in unsatisfactory preservation of the cytoplasm of the cells after the long and somewhat drastic labelling procedure although the membranes of the mycoplasma cells were appa rently intact and carried a uniform antibody layer decorated with ferritin In contrast fixation with 3 per cent glutaraldehyde caused a partial or total inhibition of antibody binding ability as visualized by the appearance of a patchy instead of a uniform extracellular coat labelled with ferritin

The ferritin particles of the second step reagent were generally attached to the outer side of the layer of specific mycoplasma antibody and were scattered around the cell outline The lack of uniformity of this arrangement certainly does not reflect a distribution of antigenic sites on the mycoplasma membranes since the layer of first step antibody as mentioned is very uniform Higher concentrations of ferritin label in the incubation mixtures might have resulted in a more uniform organization of the particles Possibly also lateral movement of rabbit antibody ferritin label complexes in the plane of the mycoplasma membrane can take place (23) explaining in part the uneven distribution of label However the results show that care should be exercised in drawing conclusions about the detailed organization of antigenic sites on mycoplasma membranes on the basis of indirect labelling methods as has also been pointed out in the case of

antiserum resulted in heavy and apparently rather uniform labelling of the organisms (12)

The membrane in the bleb region of *M gallisepticum* cells did not differ from the rest of the cytoplasmic membrane with respect to antibody binding and ferritin labelling The membrane in this region has been shown to be the site of ATPase activity (18) and to possess a particular affinity of adherence to some mammalian cells (24) However the distribution of negative charge on the membrane is uniform or at least not especially located in the bleb region (21)

The procedure used in this study showed that all the different morphological forms of *M gallisepticum* also apparently old and damaged cells were coated with rabbit antibody and ferritin label thus demonstrating the homogeneous distribution of antigens in a mycoplasma population

The method of indirect immunoferritin labelling used in this study appears to be well suited for the purpose In particular it was found advantageous to have the mycoplasmas embedded in small agar blocks during the whole of the procedure since this rendered superfluous the necessity of several centrifugations following the different incubation and washing steps and consequently allowed the experiments to be carried out with minimal amounts of reagents The absence in general of ferritin particles in the space between cells shows that the diffusion rate of the large ferritin IgG complex utilized as label was high enough to effect the removal of unbound label by washing The specificity of ferritin labelling was likewise demonstrated by the absence of labelling of protein debris in membrane preparations and by the absence of labelling of membranes incubated with tris buffer instead of the first step rabbit antiserum On the other hand the reaction of *M gallisepticum* with antiserum was not entirely specific in the sense that floccules of material adhering to the cell membranes were observed when the cells were incubated with preimmune serum as the first step reagent These patches consisted of rabbit immunoglobulin as shown by their labelling with the ferritin reagent but the unspecific attachment was easily distinguished from the specific antibody binding which was much heavier and almost entirely uniform Unspecific absorption of serum protein, in particular γ-globulin to *M gallisepticum* cells has been reported previously (4)

Membrane experiments The distinct immunological labelling of sealed membranes or empty cells in membrane fractions of *M gallisepticum* is in strong contrast to the appearance of unsealed membranes or membrane fragments in the same fractions which although covered with an amorphous layer of material did not bind the ferritin conjugate It thus seems that fragmentation of membranes has resulted in loss of antibody binding capacity the material adherent to these fragments probably being derived from the cell contents Since only one gentle washing of the lysed unfixed cells in 0 15 M NaCl was applied the step in the lysis procedure most likely responsible for the loss of membrane antigenicity in the case of unsealed membranes is the osmolysis in distilled water *M gallisepticum* membranes isolated after digitonin lysis have been shown to retain a high immunogenic potency as

homogenous membrane preparations in immunological studies in which only average properties of membranes are detected

Growth inhibition experiments The observation of destroyed cells with single-layered membranes and no antibody coat in the interior of ›break through‹ colonies and intact cells with heavy antibody coat in the periphery of such colonies and in microcolonies possibly illustrates the sequence of events in the growth inhibition reaction viz the mycoplasmas are first covered with antibody subsequently a gradual destruction of cells takes place leading to dissolution of the outer antibody coated layers of the cytoplasmic membrane The appearance of a single layered cell limitation is particularly interesting This phenomenon is not observed for instance in cells in very old cultures or in membrane fractions prepared by different procedures nor is it generally observed in badly fixed

specimens of cells or membranes It is not unlikely that the binding of antibody to the outer layers of a triple-membrane makes these more easily soluble

The detailed mechanisms of the serological growth inhibition reaction of mycoplasmas are still unexplained However, it would appear from the ultrastructural investigations of cells in »break-through« colonies that mycoplasmas are able to divide even when heavily coated with specific antibody, but that the time such cells remain alive and intact is shorter than the normal life time of agar grown mycoplasma cells The »break through« colonies are certainly not populations of mutants incapable of binding antibody specific for a parent form A mutant of *A laidlawii* resistant to immune lysis by complement but with unaltered antibody specificity has recently been characterized (6)

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HAEMAGGLUTINATION BY *STAPHYLOCOCCUS SAPROPHYTICUS* AND OTHER STAPHYLOCOCCAL SPECIES

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Hovelius B & Mårdh P A Haemagglutination by *Staphylococcus saprophyticus* and other staphylococcal species Acta path microbiol scand Sect B 87 45–50 1979

Staphylococcus saprophyticus was found to differ from *Staphylococcus epidermidis* and *Staphylococcus aureus* by its ability to agglutinate sheep erythrocytes On testing 30 strains of each species 29 strains of *S saprophyticus* and one strain each of the other two species caused agglutination Twenty-eight of 30 strains of *Staphylococcus cohnii* and *Staphylococcus xylosus* failed to cause haemagglutination The haemagglutinating activity of *S saprophyticus* when using a 10 per cent bacterial suspension was demonstrated in dilutions of 1 2–1 32 It was reduced twofold at most when exposing the bacteria to 56° C for 30 minutes while no agglutination could be demonstrated after treatment for 10 minutes at 86° C No haemagglutination could be demonstrated after treatment of the bacteria with a 5 per cent solution of trypsin Treatment of *S saprophyticus* with 0 1 M EDTA did not affect the haemagglutinating activity whereas exposure of the bacteria to 10 per cent trichloroacetic acid reduced the activity The haemagglutination was D mannose resistant and it was inhibited by homologous rabbit antiserum The agglutinates dispersed when heated at 45–56°C for 30 minutes A few of the strains of *S saprophyticus* tested also agglutinated human bovine and guinea pig erythrocytes

Key words *Staphylococcus saprophyticus* coagulase negative staphylococci haemagglutination

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Bacteria of certain species can cause direct agglutination of human and other animal erythrocytes (13) In some species e g *Escherichia coli* and *Neisseria gonorrhoeae* haemagglutination has been associated with the occurrence of fimbriae (1 10 15) while in some other species such agglutination has been ascribable to other mechanisms In contrast to fimbrial haemagglutination this other type of agglutination is usually mannose resistant and dispersed when exposed to temperature over 45° C (2)

The aim of the present study was to study the ability of *Staphylococcus saprophyticus* *S aureus* *S epidermidis* *S cohnii* and *S xylosus* to agglutinate human and animal erythrocytes The capacity of *S saprophyticus* to haemagglutinate after treatment with heat EDTA trichloroacetic acid and trypsin was also studied as was the influence on the haemagglutination of heating EDTA and D-mannose Finally haemagglutination inhibition tests using rabbit antisera were performed

MATERIALS AND METHODS

Bacteria and Culture Technique

Thirty strains of *S. aureus* isolated from pus and 30 strains each of *S. epidermidis* and *S. saprophyticus* isolated from voided urine specimens were tested. In addition tests were made on 15 strains each of *S. cohnii* and *S. xylosus* isolated from urethral specimens. The staphylococci tested had been classified according to Kloos & Schleifer (9). Two of the strains of *S. epidermidis* were resistant to 2µg/ml of novobiocin while the other strains of this species were susceptible to this concentration.

The bacteria studied were inoculated into tryptone broth (Oxoid) and incubated at 37° C overnight. Also staphylococci grown at 37° C overnight on a medium consisting of Blood agar base No. 2 (Oxoid) with 4 per cent defibrinated horse blood were used.

Erythrocytes

One per cent suspensions of fresh washed human (blood groups AB Rh+ and O Rh+), guinea pig, ox and sheep (3 animals) erythrocytes in physiological saline were used.

Treatment of Bacteria

Before use the bacterial suspensions were centrifuged at 3000×g for 5 minutes and suspended in phosphate buffered saline (PBS) pH 7.2. This procedure was repeated once. If not otherwise stated 10 per cent (v/v) bacterial suspensions in PBS were used.

Forty per cent (v/v) suspensions in PBS of four strains of *S. saprophyticus* that caused haemagglutination of sheep erythrocytes and of four non-agglutinating strains of *S. epidermidis* were heated at 37°C, 45°C, 56° C and 86° C for 10 and 30 minutes, respectively. The bacteria used had been cultured in tryptone broth. Other aliquots of these eight suspensions were mixed with an equal amount of 0.1 M disodium ethylene diamine tetraacetate (EDTA) or 10 per cent (v/v) trichloroacetic acid (TCA) at 4° C for 1 hour. The four strains of *S. saprophyticus* were also exposed to an equal amount of a 5 per cent solution of bovine pancreas trypsin type III (Sigma) at 37° C for 20 minutes. All mixtures were centrifuged, washed four times in PBS and re-suspended to the original concentration. Untreated bacteria in PBS were used as controls.

Ten per cent suspensions (v/v) of 20 strains of *S. saprophyticus* in PBS stored at 4° C for 2 weeks were harvested by centrifugation and used in haemagglutination tests.

Supernatants of Staphylococcal Broth Cultures

In some experiments the supernatants obtained by centrifugation as described above of 1- and 3-day-old broth cultures of *S. saprophyticus* and *S. epidermidis* were used in haemagglutination tests. Supernatants obtained by centrifugation of the 20 cultures of *S. saprophyticus* stored for 2 weeks were also tested.

Haemagglutination Tests

Twenty five µl of the bacterial suspensions were added to 25 µl of the erythrocyte suspensions in wells of disposable plastic microtitration plates (Flow Laboratories Ltd.). The plates were sealed with cellophane, shaken and incubated at room temperature for 2 hours before reading.

Haemagglutination tests with sheep erythrocytes were also made on glass slides. The tests were read after being kept for 5 minutes at room temperature. Bacteria harvested from blood agar plates were also used in these two haemagglutination test runs.

Twofold serial dilutions of 10 per cent suspensions of five strains of *S. saprophyticus* were made in PBS in microtitration plates. In each well 25µl of the bacterial suspensions were mixed with 25 µl suspension of the human, sheep, ox or guinea pig erythrocytes.

The occurrence of agglutinate was determined by inspection of plates and glass slides by the naked eye.

Haemagglutination Tests with Sheep Erythrocytes in the Presence of EDTA and D-mannose

Four of the strains of *S. saprophyticus* were exposed to increasing concentrations of EDTA. Twofold serial dilutions of the 40 per cent bacterial cell suspension, 25 µl in each well, were made in PBS using microtitration plates. To each dilution 25 µl of either 0.1, 0.5, 1.0, 5.0 or 10.0 mM EDTA were added. These suspensions were used in agglutination tests with sheep erythrocytes.

Tests were also performed in the presence of 2 per cent D-mannose (Merck). Four strains of *S. saprophyticus* were tested using a 10 per cent bacterial suspension which was twofold serial diluted in PBS. Twenty five µl of the suspension were mixed with 25 µl D-mannose and 25 µl of the sheep erythrocytes.

Influence of Temperature on Haemagglutination

Agglutinates of sheep erythrocytes and 10 per cent bacterial suspensions of four strains of *S. saprophyticus* performed in microtitration plates were stored at 4° C, 45° C and 56° C for 30 minutes and thereafter re-examined.

Preparation of Antisera

Antisera against one strain each of *S. epidermidis* and *S. saprophyticus* were prepared in rabbits. The bacteria used had been grown in tryptone broth, formalin treated, washed three times in saline and suspended in the saline to form a 10 per cent suspension. The rabbits were injected intravenously weekly for 3 weeks with 1 ml of the suspension and boostered after 1 and 2 months. The rabbits were ear bled 2, 5 and 12 weeks after the first injection. The sera were heated at 56° C for 30 minutes and absorbed with a 50 per cent suspension of sheep erythrocytes before use.

The titre of agglutinating antibody to *S. saprophyticus* in the rabbit sera collected 12 weeks after the immunization with *S. saprophyticus* was 1:512, while that of the rabbit immunized with *S. epidermidis* was 1:32.

Haemagglutination Inhibition Tests

Pre immune and immune sera from two rabbits were used in haemagglutination inhibition tests with *S saprophyticus* All sera collected from each rabbit were serially diluted in PBS Twenty five µl of the diluted sera and the same volume of a 5 per cent suspension of the strain of *S saprophyticus* used for immunization were mixed in wells of microtitration plates which were incubated at 37° C for 30 minutes Thereafter 25 µl of the suspension of sheep erythrocytes were added After 2 hours incubation at room temperature the tests were read In control experiments, PBS was used instead of serum

RESULTS

Agglutination of Various Erythrocytes

Sheep erythrocytes All but two of the 30 strains of *S saprophyticus* grown in tryptone broth agglutinated erythrocytes from two of the sheep used while 25 of the strains caused agglutination of erythrocytes from the third sheep studied (Table 1) The five strains which did not agglutinate erythrocytes of the third sheep included the two strains which did not agglutinate erythrocytes of the other two sheep Only one strain each of *S epidermidis* (novobiocin susceptible) and *S aureus* agglutinated erythrocytes of any of the three sheep *S saprophyticus* harvested from blood agar plates never caused haemagglutination

In comparable experiments no differences in the test results were obtained when using microtitration plates and glass slides

Two of the 15 strains of *S xylosus* caused haemagglutination whereas none of the 15 strains of *S cohnii* did so

Guinea pig bovine and human erythrocytes Six of the 30 strains of *S saprophyticus* agglutinated guinea pig and bovine erythrocytes Four of these six strains also agglutinated human erythrocytes One strain each of *S aureus* and *S epidermidis* agglutinated erythrocytes from all the animal and humans tested (Table 1)

The highest dilutions of five strains of *S saprophyticus* which caused agglutination of human, sheep, guinea pig and ox erythrocytes, are shown in Table 2

Haemagglutination by Four Strains of S saprophyticus *after Various Treatment of the Bacteria*

Heat S saprophyticus heated to 86° C for 10 minutes did not agglutinate sheep erythrocytes The haemagglutinating activity was reduced by 1 to 2 steps of dilution after treatment of one of the strains tested at 56° C for 30 minutes, but not for 10 minutes The agglutination with the other three strains was not affected by this treatment The haemagglutinating activity was not affected by [illegible] haemagglutination

TCA Treatment with 10 per cent TCA reduced but did not abolish, the capacity of three of the four strains of *S saprophyticus* to form agglutinates After the treatment, haemagglutination occurred but only when using higher concentrations of the bacteria One strain was not affected by treatment with TCA

Trypsin Treatment of the strains with trypsin abolished the haemagglutinating activity completely

Storage The bacterial suspensions of S saprop-

TABLE 1 *Direct Haemagglutination of Human Sheep Guinea Pig and Ox Erythrocytes by 30 Strains of* Staphylococcus aureus Staphylococcus epidermidis *and* Staphylococcus saprophyticus[a]

Erythrocytes used	No of strains causing agglutination		
	S aureus N = 30	*S epidermidis* N = 30	*S saprophyticus* N = 30
Human			
AB Rh +	1	1	4
O Rh +	1	1	4
Sheep			
I	1	1	28
II	1	1	25
III	1	1	28
Guinea pig	1	2	6
Ox	1	1	6

[a] 10 per cent bacterial suspensions were used

TABLE 2 *Direct Haemagglutination of Human Sheep (2 Animals) Guinea Pig and Ox Erythrocytes by Five Strains Each of Staphylococcus saprophyticus, Staphylococcus* aureus *and* Staphylococcus epidermidis[a] *Reciprocals of the Highest Dilutions Causing Agglutination are given*

Species tested	Strain no	Human		Sheep		Guinea pig	Ox
		AB Rh +	*O, RH +*	*I*	*II*		
S saprophyticus	1	–[b]	–	2	16	4	–
	2	8	4	32	32	4	8
	3	–	–	2	16	2	–
	4	8	4	16	16	8	8
	5	–	–	16	32	32	–
S aureus	1–5	–	–	–	–	–	–
S epidermidis	1–5	–	–	–	–	–	–

[a] 10 per cent bacterial suspensions were diluted twofold in phosphate buffered saline pH 7 2
[b] < 2

hyticus kept in 4° C for 14 days still caused haemagglutination

Haemagglutination Tests Using Supernatants of Staphylococcal Cultures

No agglutinating activity could be demonstrated when testing the supernatants from *1*- and *3* day-old broth cultures of *S saprophyticus* and *S epidermidis* Nor possessed the supernatants of cultures stored for 14 days any such activity

Haemagglutination Tests in the Presence of EDTA and D-mannose

The haemagglutination of the four strains of *S saprophyticus* tested was not affected by the presence of 0 1–10 mM EDTA Nor was the haemagglutinating activity affected by the presence of D-mannose

Influence of Temperature on Haemagglutination

Agglutinates of sheep erythrocytes and *S saprophyticus* were not affected when stored at 4° C for 30 minutes The agglutinates dispersed when exposed to 45° C for *30 minutes in three of four strains* tested This also occurred with all four strains when heated at 56° C for the same period of time

Haemagglutination-Inhibition Tests

The pre-immune serum from the rabbit immunized with *S saprophyticus* inhibited haemagglutination by *S saprophyticus* at a titre of 1 4 as did the serum drawn 2 weeks after the first immunization The sera drawn 5 and 12 weeks after the first immunization caused haemagglutination inhibition at a titre of 1 32 The pre immune and immune sera from the rabbit immunized with *S epidermidis* inhibited the agglutination at the same low titre viz 1 4

DISCUSSION

The ability of most strains of *S saprophyticus* to agglutinate sheep erythrocytes distinguishes them from most strains of the other staphylococcal species tested, including those of the two other coagulase-negative novobiocin-resistant species viz *S cohnii* and *S xylosus* The three species mentioned have been distinguished from other [illegible] their resistance [illegible] we also have [illegible] *epidermidis* [illegible] all isolated from hospitalized patients, to be resistant *to this concentration of novobiocin (5) The two* novobiocin-resistant strains of *S epidermidis* tested in this study did not cause haemagglutination

One of the few practical applications of bacterial haemagglutination reported is that by *Finkelstein & Mukerjee* (4) who by such tests distinguished El Tor vibrios from other *Vibrio cholerae* Haemagglutination can also be used to separate *S saprophyticus* from other staphylococcal species provided that they have been cultured in liquid medium such as tryptone broth but not when using strains harvested from blood agar plates

Direct haemagglutination caused by certain bacterial species has been associated with filamentous appendages viz fimbriae This type of haemagglutination has been shown to occur in *E coli* and to be mannose sensitive (2) Filamentous appendages are not known to occur in staphylococci On scanning

and transmission electron micrographs we were not able to detect any difference in surface structures of strains of *S saprophyticus* and *S epidermidis* used in the agglutination tests

The haemagglutinating activity of *S saprophyticus* after treatment with EDTA or TCA was not obviously affected It has been suggested that such treatment of fimbriated bacteria strips off the pili, thereby inhibiting their haemagglutinating ability (3)

Bacteria can cause agglutination of erythrocytes of certain animal species, but agglutinate not at all or less readily erythrocytes of other species (13) With a few exceptions, the tested strains of *S saprophyticus* caused agglutination of sheep erythrocytes while only some of the same strains agglutinated erythrocytes of the other animals studied The reason for these species differences is not known

In *in vitro* experiments, we found that *S saprophyticus* had a more pronounced capacity to adhere to human urothelial cells than had *S epidermidis* (12) *E coli* K88, has a pronounced capacity to adhere to epithelial cells of the jejunum of pigs (7) Th s bacterium [illegible] ability to adhere and to agglutinate has been suggested to be mediated by the same protein antigen (8) The K88 antigen causes mannose-resistant haemagglutination and the agglutinates disperse when exposed to heat like *S saprophyticus* [illegible] urinary tract infections caused by *S saprophyticus* Aggregates of erythrocytes and cocci are a common finding in urine sediments of such patients (6)

S saprophyticus is also often found to be adhered to urothelial cells In preliminary tests, such organisms harvested direct from the urine of women with urinary tract infections usually caused haemagglutination This implies that the haemagglutinating activity of *S saprophyticus* may be present *in vivo*

S saprophyticus lacks coagulase desoxyribonuclease phosphatase protein A, and haemolytic ability (14) characteristics discussed as possible pathogenicity factors in *S aureus* Whether or not the capacity of *S saprophyticus* to haemagglutinate expresses a virulence factor remains to be determined

Haemagglutination by *Fusobacterium nucleatum* (3) is Ca^{++}-dependent. The haemagglutination caused by *S saprophyticus* was not affected by the presence of EDTA The haemagglutinating activity of *S saprophyticus* was destroyed when the bacterium had been heated at 86° C, but was only slightly affected when it was exposed to 56° C Furthermore, treatment of *S saprophyticus* with trypsin abolished the haemagglutinating activity This suggests the haemagglutinin to be a protein

Sera of the rabbit immunized with *S saprophyticus* inhibited the haemagglutination of *S saprophyticus* while sera of the rabbit immunized with *S epidermidis* did not do so This suggests that the haemagglutinin may act as an antigen

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TABLE 2 *Direct Haemagglutination of Human Sheep (2 Animals) Guinea Pig, and Ox Erythrocytes by Five Strains Each of* Staphylococcus saprophyticus, Staphylococcus aureus *and* Staphylococcus epidermidis[a] *Reciprocals of the Highest Dilutions Causing Agglutination are given*

Species tested	Strain no	Human AB, Rh +	Human O, RH +	Sheep I	Sheep II	Guinea pig	Ox
S saprophyticus	1	–[b]	–	2	16	4	–
	2	8	4	32	32	4	8
	3	–	–	2	16	2	–
	4	8	4	16	16	8	8
	5	–	–	16	32	32	–
S aureus	1–5	–	–	–	–	–	–
S epidermidis	1–5	–	–	–	–	–	–

[a] 10 per cent bacterial suspensions were diluted twofold in phosphate buffered saline, pH 7 2
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DISCUSSION

The ability of most strains of *S saprophyticus* to agglutinate sheep erythrocytes distinguishes them from most strains of the other staphylococcal species tested, including those of the two other coagulase-negative, novobiocin-resistant species, viz *S cohnii* and *S xylosus* The three species mentioned have been distinguished from other coagulase negative staphylococci by their resistance to 2 µg/ml of novobiocin However, we also have found 10 per cent of 150 strains of *S epidermidis* all isolated from hospitalized patients, to be resistant to this concentration of novobiocin (5) The two novobiocin-resistant strains of *S epidermidis* tested in this study did not cause haemagglutination

One of the few practical applications of bacterial haemagglutination reported is that by *Finkelstein & Mukerjee* (4), who by such tests distinguished El Tor vibrios from other *Vibrio cholerae* Haemagglutination can also be used to separate *S saprophyticus* from other staphylococcal species provided that they have been cultured in liquid medium, such as tryptone broth, but not when using strains harvested from blood agar plates

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COMPLETE AND INCOMPLETE Ibc PROTEIN FRACTION IN GROUP B STREPTOCOCCI

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Bevanger L & Mæland J A Complete and incomplete Ibc protein fraction in group B streptococci Acta path microbiol scand Sect B 87 51–54 1979

The Ibc protein fraction of group B streptococci was isolated from the type Ib strain H36B the type Ic strain A909 and the type Ic strain 335 and examined against antisera by immunodiffusion and immunoelectrophoresis The fraction from the Ib strain and one of the Ic strains (A909) contained two antigens or groups of antigens called the α and β antigens respectively Strain 335 produced the α but not the β antigen This was also the case with three other group B isolates previously classified as type Ic One type II and one type III strain produced both antigens The results suggest that group B streptococci producing the Ibc fraction can be subdivided further on the basis of the antigens that are present in that fraction

Key words Group B streptococci Ibc protein fraction complete and incomplete

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Division of group B streptococci into serotypes Ia Ib II or III is based on specific carbohydrate antigens produced by the bacteria Group B streptococci may also produce the protein antigen originally called the Ic protein by *Wilkinson & Eagon* (6) and later designated the Ibc protein antigen by *Wilkinson* (5) The antigen was prepared by extraction of whole cells of the bacteria with hot HCl and separated from the polysaccharides present in the extract by precipitation with cold trichloroacetic acid (6) On immunodiffusion the Ibc protein preparation produced two precipitation lines against antisera (6) Probably all type Ib strains and some type II and type III strains possess the protein antigen (2 4 7 8) Strains that produce both the carbohydrate antigen Ia and the Ibc protein are currently classified as serotype Ic (5 6 9)

Recent experiments showed that the fluorescent antibody test can be used for rapid identification of those group B strains of the carbohydrate antigen types Ia II and III that produce the Ibc protein antigens (2) Such strains showed cross reactivity with the anti Ib conjugate This report presents the results of a study designed to compare the Ibc protein fractions from different group B strains

MATERIAL AND METHODS

Strains

The following type strains of group B streptococci were used Type Ia 090 type Ib H36B type Ic A909 type II V8 type II 18RS21 and type III D136C Other strains used were the clinical isolates of group B streptococci described previously (2) in particular the type Ic strain 335 All strains were kept at −80°C in Stuart transport medium and thawed immediately before culturing

Culture

Todd Hewitt broth modified by increasing the concentration of Na_2HPO_4 and glucose eight times (1) was inoculated with the bacteria and incubated at 37°C for 18 h under constant agitation The streptococci were collected by centrifugation at 8000 × *g* for 10 min and washed once in phosphate buffered saline pH 7 2 (PBS)

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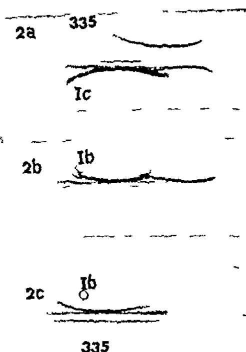


Fig 2 Immunoelectrophoresis Antigen wells were filled with the Ibc fraction from the type Ic strains 335 and A909 respectively or the type Ib strain H36B as indicated Serum basins contained anti Ib (2a and 2b) or anti Ib absorbed with extract from strain 335 (2c) Anode to the right

anti 335 serum was tested against the extracts only the α line was produced (Fig 1c)

Absorption of the antisera with the Ib or A909 extract eliminated all antibody activity detectable by the precipitation test Absorption with the 335 extract or with bacteria of this strain resulted in removal of the antibody to the α antigen but not to the β antigen

Th ...

...an antigen with a comparatively fast anodic migration was present in all three extracts The A909 and Ib extracts but not the 335 extract also produced a second line with slower anodic migration frequently in the form of a cluster of lines Presumably the two antigens or groups of antigens corresponded to the α and β lines observed in the agar precipitation test

Digestion of the extracts with pepsin resulted in removal of the precipitation with antisera Trypsin digestion eliminated the formation of the β line but did not affect the α line The α and β lines formed as before when the extracts were oxidized with periodate and then examined

Ibc Protein Fraction from Other Strains of Group B Streptococci

Extract was prepared form other isolates of group B streptococci and tested by agar precipitation and immunoelectrophoresis against the antisera From each of the serotypes Ia II and III one strain that did not show cross reactivity with the FITC anti Ib conjugate was studied Precipitation with the antisera was not observed when using extract from any of these strains Extract prepared from a type II and a type III strain that showed FA staining by the conjugate produced both the α and β lines on testing with the antisera On the other hand extract from each of three type Ic strains produced the α line but not the β line similar to the strain 335

DISCUSSION

The Ibc protein fraction from the type strains Ib (H36B) and Ic (A909) contained two precipitinogens here called the α and β antigens respectively The β antigen was susceptible to trypsin and pepsin the α antigen to pepsin only These findings are similar to the observations of *Wilkinson & Eagon* (6) working with the same strains

The β antigen gave rise to a single line in the agar gel diffusion test but showed a more complex pattern of lines on immunoelectrophoresis It has not been established as yet whether this was due to the presence of different antigens with similar electrophoretic mobility or to a heterogeneity of one and the same antigen possibly induced by the harshness of the ...

The demonstration in this study of the α and β ...

... type ... 335 this fraction contained the α but not the β antigen Moreover antibody to the β antigen could not be detected in the anti 335 serum and absorption of antisera with extract or bacteria of this strain failed to remove antibody to the β antigen It must be concluded that the β antigen was not produced by strain 335 which accordingly produced an incomplete Ibc protein fraction

Group B isolates of the serotype Ic produce both the carbohydrate antigen Ia and the Ibc fraction (5 6 9) type II ...

(...

8

Extraction

The Ibc protein fraction was isolated from whole cells of the streptococci essentially as reported by *Wilkinson & Eagon* (6) The bacteria were extracted with 0 2 N HCl at 50°C for 2 h and then removed by centrifugation at 8000 × *g* for 10 min Proteins were precipitated from the *supernatant fluid* with four volumes of 10 per cent trichloroacetic acid (TCA) at 4°C for 20 h The pellet obtained after centrifugation was dissolved in 0 15 M NaCl and precipitated *again with an equal volume of 10* per cent TCA The final precipitate was dissolved in PBS *and dialyzed* against water Undissolved material was then removed by centrifugation and the solution (the Ibc protein fraction) was lyophilized

Antisera

Antiserum against whole cells of the type Ib strain (H36B) and the type Ic strains A909 and 335 anti Ib A909 and 335 respectively was prepared by immunization of rabbits as reported earlier (2) The antisera were used unabsorbed after adsorption with whole cells of the bacteria (2) or with the Ibc fraction One mg or more of this fraction was added to 0 2 ml of antiserum the mixture was kept at 4°C for 20 h and centrifuged at 8000 × *g* for 10 min the supernatant was used in the tests

Fluorescent Antibody (FA) Test

Immunoglobulin from the anti Ib serum conjugated *with fluorescein isothiocyanate (FITC) was used* The preparation method and data characterizing the conjugate have been described earlier (2) Strains of group B streptococci were examined by the FA test as described (2)

Immunodiffusion and Immunoelectrophoresis

The immunodiffusion test was performed as before (2) using slides prepared with 1 per cent agarose (Noble Difco) in PBS For the immunoelectrophoresis 1 per cent agaose in 0 03 M veronal buffer pH 8 6 was employed The electrophoresis was run for 90 min at a constant voltage of 10 V per cm in 0 05 M veronal buffer pH 8 6 Unabsorbed or absorbed antiserum was then applied and the slides were kept at 4°C for two days Antigen in a concentration of 1 mg per ml was used for the agar precipitation test and 8 mg per ml for the immunoelectrophoresis The slides were washed dried and stained with Coomassie Brilliant Blue as recommended (3)

Enzymic Digestion and Periodate Oxidation

Digestion of materials with trypsin (B grade Calbiochem) was carried out in a 0 05 M tris HCl buffer pH 8 0 and with pepsin (B grade Calbiochem) in a 0 05 M citrate phosphate buffer pH 2 6 at 37°C for 4 h with a substrate to enzyme ratio of 4 1 (w/w) The mixtures were then dialyzed against water and lyophilized Material 4 mg per ml was dissolved in a 0 25 per cent (w/v) solution of Na *meta*periodate in PBS The mixture was kept in the dark at 20°C for 20 h dialyzed against water and lyophilized

RESULTS

FA Test

The effect of absorption of the FITC conjugate against the type strain Ib (H36B) with bacteria of type Ic strains A909 or 335 was examined Absorption with strain A909 eliminated the FA staining of both the type Ic strains On the other hand absorption with strain 335 did not eliminate the staining of strain A909 The results suggest that the Ibc protein fraction of strain 335 may not be identical with that of strain A909 or the type Ib strain This possibility was tested by examination of the Ibc fraction isolated from bacteria of these strains

Immunodiffusion and Immunoelectrophoresis

Testing of the Ibc protein fraction from each of the three strains against the anti Ib serum (Fig 1a) resulted in a precipitation pattern similar to that obtained with the anti A909 serum (Fig 1b) A continuous precipitation line the α line was caused by an antigen present in all three extracts A second line the β line was produced by an antigen in the Ib *and A909 extracts but not by the 335 extract* When

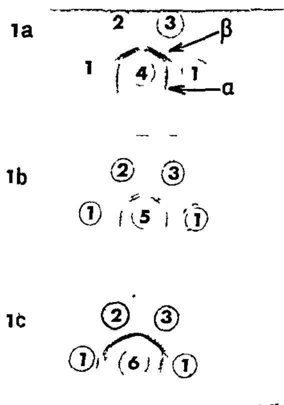


Fig 1 Immunodiffusion Peripheral wells contain the Ibc protein fraction from the type Ic strain *335 (1)* the type Ic strain A909 (2) and the type Ib strain H36B (3) Central wells contained anti Ib (4) anti A909 (5) and anti 335 (6) α and β see text

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SEROLOGY OF *NEISSERIA GONORRHOEAE* DEMONSTRATION OF STRAIN-SPECIFIC ANTIGENS BY IMMUNOELECTROPHORESIS, IMMUNOFLUORESCENCE AND CO-AGGLUTINATION TECHNIQUES

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Danielsson D & Sandstrom E Serology of *Neisseria gonorrhoeae* Demonstration of strain specific antigens by immunoelectrophoresis immunofluorescence and co agglutination techniques Acta path microbiol scand Sect B 87 55–64 1979

[illegible] with antigen containing intermediate agar gel as a two peak precipitin line one peak close to the antigen well the other towards the anode A similar pattern was found for each of the four strains studied These st[illegible] rocket line immunoelectrophoresis (R LIE) tests w[illegible] Using whole cells strain specific antigens were also [illegible] od cross absorbed antibodies The results correspc[illegible] Immunosorption experiments indicated identity between the strain-specific antigens shown by COA tests and those demonstrated by C LIE and R LIE tests

Key words *Neisseria gonorrhoeae* strain specific antigens co agglutination immunoelectrophoresis indirect immunofluorescence

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Serological investigations with the aim of classifying *Neisseria gonorrhoeae* have shown these organisms to be complex and heterogenous from an antigenic point of view (cf ref 11 14 23) However common as well as »group« »type« or »strain« specific antigens were demonstrated in studies of viable or killed whole cells by agglutination (27 30) complement fixation (22) immunofluorescence (8 21 29) and bactericidal tests (13 28) and in studies of disintegrated organisms or antigenic fractions in various stages of purification by agar gel precipitation (1 2 8 12 14) and passive haemagglutination or haemolysis (5 18 19) Very few of these studies correlated the serological findings og whole cells with those of disintegrated organisms or antigenic fractions

We described recently use of the so-called co agglutination technique for the serological identification of *Neisseria gonorrhoeae* (10) In the present study we used this technique in combination with indirect immunofluorescence to demonstrate strain specific antigens of viable or killed whole gonococcal (GC) organisms These findings correlated well

type II or type III strains has not as yet been established, although this has been proposed (2) The strain 335 used in this study was classified previously as type Ic, due to FA staining of the bacteria both by the anti-Ia and anti-Ib FITC conjugates (2) Staining by the anti-Ib conjugate can be explained on the basis of antibody to the α antigen produced by this strain However, it is debatable whether this strain and others that produce an incomplete Ibc fraction should be classified as serotype Ic The results of this study suggest the possibility of further serological division of the Ibc protein producing group B strains according to the antigens of this fraction Out of a total number of eight Ibc producing strains, four strains produced an incomplete fraction, and all of these were previously classified as serotype Ic However experiments to date have not revealed the actual frequency of an incomplete Ibc fraction in group B streptococci, or the impact that this observation may have on the classification of the bacteria

The technical assistance of Mrs *Augusta Nass* is greatly appreciated

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µl of the supernatant of disrupted GC organisms (200 mg ml⁻¹ of the wet weight see above) in the antigen well The reference gel contained 8 µl per cm² of the undiluted anti GC antiserum The second dimension electrophoresis was run overnight with a potential gradient of 2 V cm⁻¹ The antigen wells were filled correspondingly for R LIE and with corresponding amounts of anti GC antiserum in the reference antibody gel The intermediate gels with antigen contained 4–5 mg disrupted GC cells per cm² and those with absorbed anti GC antiserum 16 µl per cm²

After electrophoresis the plates were washed over night in saline then for 10 min in distilled water pressed under moist filter paper and soft blotting paper dried and finally stained with amido black or Coomassie brilliant blue (3)

Co agglutination (COA)

The production and stabilization of protein A containing staphylococci to make COA reagent followed the procedures described by *Kronwall* (17) For coating of the staphylococci with antibodies 1 ml of a 10% suspension (w/v based on the sediment obtained after centrifugation at 2 500 *g* for 15 min) was added to 0 1 ml of undiluted or twofold diluted unabsorbed or absorbed rabbit anti GC antiserum After mixing the suspension the staphylococci were washed twice and suspended for 1% (v/v) in PBS containing 0 1% (w/v) sodium azide In this way 10 ml reagents were obtained from 0 1 ml serum Control reagent consisted of staphylococci uncoated or coated with gammaglobulin from normal rabbit serum The suspensions were stable for at least 3–4 months when stored at +4° C

COA tests were performed with GC organisms grown for 18–20 h on CMT medium One drop of a 1% (w/v wet wheight after centrifugation at 2 500 *g* for 15 min) suspension of GC cells in PBS or 3–6 colonies collected with a loop was mixed thoroughly on a glass slide with one or two drops of reagent staphylococci The slide was then tilted approximately once a second for 2 min and the reaction was read in oblique light COA tests were carried out in the same way with a 1% (w/v) suspension of GC cells treated by heat at 100° or 120° C for 60 min or with the supernatant or the sediment (after centrifugation at 15 000 *g* for 30 min) of cells sonicated as described above Negative reactions were graded or ± weak reactions + moderately strong + + and strong or very strong + + + The + + and + + + reactions usually started to appear within one min and gradually became stronger within the next minute The + reactions usually took 2 min to appear

Indirect Immunofluorescence (IFL)

IFL tests were carried out with whole untreated GC cells or with cells treated by heat at 100° C or 120° C GC cells at an approximate concentration of 5 × 10⁷ ml were placed on a circular area (about 1 cm) on ordinary microscopic glass slides dried in air and gently fixed by heat Unabsorbed or absorbed anti GC antiserum was diluted twofold with a starting dilution of 1 10 One drop (approximately 0 03 ml) of each dilution was placed on the cells The slides were then kept at room temperature in a moist chamber for 20–30 min and washed twice (2×5 min) in PBS after which fluorescein isothiocyanate labelled sheep anti rabbit IgG (Calbiochem) was applied This conjugate had a weight F/P ratio of 8 5 × 10⁻³ with an antibody concentration of 1 8 mg ml⁻¹ It was used at a dilution of 1 20 as ascertained by checkerboard titration (4) After a reaction time of 20–30 min the slides were washed and mounted under cover glasses in the usual way Microscopy was carried out with a Zeiss RA II fluorescence microscope equipped with an Osram HBO 200 high pressure mercury lamp and a dark field condensor BG3 was used as primary and Zeiss 47 as secondary filters The tests were read at a magnification of 400 × and the reactions were graded as − 1+ 2+ 3+ or 4+ as described previously (7 8) Reactions read as 3+ or 4+ were considered positive

RESULTS

Crossed Immunoelectrophoresis

Examples of CIE tests of GC antigens with their homologous rabbit antisera are shown in Figures 1 & 2 The number of precipitin lines was dependent on the antiserum used Three to four and up to 20 lines were formed with some of the homologous GC antigen antibody combinations and numbers of bands varying between these extremes with others Antisera from rabbits immunized with antigen in Freund s adjuvant regularly gave more precipitin lines than those from rabbits immunized by (iv) route only Precipitin bands obtained with homologous antigens were also demonstrated with the heterologous antigens thus confirming the presence of common antigens (8 11 14 23) However regardless of ho

[illegible]

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... arding because of the complexity of the precipitin lines demonstrated C LIE and R LIE tests with antigen containing intermediate agar gel as described by *Axelsen et al* (3) were therefore performed since these techniques permit the demonstration of strain-specific antigens in complex systems The overall results of these tests are summarized in Table 1 The homologous GC strains formed a specific precipitin line with their homologous antibodies when sonicated cells of one

with the strain specific antigens demonstrated by cross line and rocket line immunoelectrophoresis of disintegrated organisms

MATERIAL AND METHODS

Neisseria gonorrhoeae strains

Four GC strains with our provisional designations GC 670 GC EJ (both of auxotypes Arg⁻ Hyx Ura) GC SOA (Pro Met) and GC 505(auxotype Zero) were used in this study (auxotyping kindly performed by Dr *I Moberg* National Bacteriology Laboratory Stockholm) These strains had been selected from a group of 17 strains on the basis of serological diversity In the following they will be referred to as strains *A B C* and *D* respectively All strains were isolated from genital specimens in 1973 at our laboratory in Örebro Sweden strain *A* from a female with disseminated gonococcal infection strain *B* from a patient with acute salpingitis and strains *C* and *D* from two males with uncomplicated gonorrhoea The infection with strain *C* was acquired in South East Asia the others in Sweden

The GC organisms were isolated and identified as described previously (9 10) Colonies were transferred after first or second subculture on the colony morphology typing medium (CMT) described by *Kellogg et al* (16) for selecting the virulent colony morphology types T1/T2 and the avirulent T3/T4 For the subsequent experiments the virulent colony types fulfilled the criteria of T2 according to *Kellogg et al* (16) while the avirulent were a mixture of T3/T4 After a minimum number of subcultures (usually 3–7 for T2 and 10–15 for T3/T4) the strains were suspended in trypticase soy broth and stored in small tubes at −63° C Small aliquots were taken from these tubes with a platinum loop or Pasteur pipette and recultured on the CMT medium when needed

Gonococcal Antisera

T2 colony morphology types of the four GC strains were cultured on the CMT medium for 18–20 h in candle jars at 35°–36° C The organisms were harvested in sterile saline treated with formalin as described previously (7) and adjusted to a concentration of approximately 4×10^9 organisms ml $^{-1}$ according to a McFarland scale Groups of eight rabbits were immunized with each of the four GC strains as follows One group of rabbits (two for each strain) was injected subcutanously with GC organisms mixed with an equal part of complete Freund s adjuvant 1 5 ml and 1 0 ml in each hind and fore leg respectively A series of four intravenous (iv) injections were given 4–5 weeks later the first dose 0 25 ml and the following doses every 3–4 days increasing to 0 5 1 0 and 2 0 ml The rabbits were bled 7–10 days after the last injection Another group of eight rabbits (two for each GC strain) were immunized by the intravenous route only with doses and time intervals as above These rabbits were given two series of injections (iv) with two weeks rest in between They were bled 7–10 days after the last injection Both antisera and preimmune sera were stored an aliquots of 1 ml at −40° C After thawing merthiolate (1 10 000) was added to the serum which was then stored at +4° C and used within a period of 4–6 months

Absorptions of antisera were performed with organisms (250 mg ml $^{-1}$ wet weight) suspended in phosphate buffered saline (PBS) pH 7 2–7 4 and disrupted by sonication with a 100 watt MSE apparatus operated at maximum efficiency as described below under C LIE and R LIE tests One volume of undiluted serum was mixed with one volume of sonicated organisms concentrated by negative pressure dialysis corresponding to a wet weight of 1000 mg ml $^{-1}$ whole cells The mixture was incubated in a water bath at 37° C for 3–4 hours and in the refrigerator overnight Absorbed antiserum was recovered by centrifugation twice at 30 000 *g* for 30 min In this way the absorbed antiserum was diluted 1 2 Additional absorptions were sometimes carried out with formalin treated whole cells One part antiserum was mixed with one part of packed cells (after centrifugation at 2 500 *g* for 15 min) incubated at 37° C for 3–4 hours and then recovered by centrifugation

Immunoelectrophoresis

Crossed immunoelectrophoresis (CIE) and crossed line and rocket line immunoelectrophoresis (C LIE and R LIE) with antigen or antiserum containing intermediate agar gel were carried out as described by *Axelsen et al* (3) using the micromodification with 5 × 5 cm glass plates An electrophoresis apparatus cooled with water at 15° C was used The barbital buffer (LKB Bromma Sweden pH 8 6 ionic strength 0 1) in the electrophoresis vessels was recirculated to minimize changes of the pH of the buffer The electrophoreses were run in 1% (w/v) agarose (Miles lot SC 3) containing LKB barbital buffer pH 8 6 ionic strength 0 025 The areas covered by reference antibody gel in CIE tests (Fig 1 and 2) were approximately 17 5 cm² and those covered by intermediate gel and reference antibody gel in C LIE and R LIE tests (Fig 3 and 4) approximately 5 and 12 5 cm² respectively

The antigens used in the immunoelectrophoresis tests were derived from cells scraped from plates containing more than 90% colony morphology type colonies The cells were suspended in barbital buffer (250 mg ml $^{-1}$ wet weight) and disrupted by sonic treatment with a 100 watt MSE ultrasonic apparatus operated at maximal efficiency for periodic intervals of 30 s The suspensions became transparent after sonication for a total of 150–210 s Smears stained by Gram or immunofluorescence techniques showed 100% disintegration of the cells Reference antigens were tested in concentrations from 12 5 mg ml $^{-1}$ – 400 mg ml $^{-1}$ (calculated from the wet weight of cells centrifuged at 2 500 *g*) and it was found that 200 mg ml $^{-1}$ gave optimal results Tests with this crude antigen gave the same precipitin pattern as the supernatant (obtained after centrifugation at 30 000 *g* for 20 min) which was subsequently used since it made interpretation of formed precipitin lines easier GC antigen treated by heat at 100° C or 120° C for 60 min was used in some of the experiments

The first dimension of CIE or C LIE was carried out with a potential gradient of 6 V cm $^{-1}$ for 45 min with 10

μl of the supernatant of disrupted GC organisms (200 mg ml^{-1} of the wet weight see above) in the antigen well The reference gel contained 8 μl per cm^2 of the undiluted anti GC antiserum The second dimension electrophoresis was run overnight with a potential gradient of 2 V cm^{-1} The antigen wells were filled correspondingly for R LIE and with corresponding amounts of anti GC antiserum in the reference antibody gel The intermediate gels with antigen contained 4–5 mg disrupted GC cells per cm^2 and those with absorbed anti GC antiserum 16 μl per cm^2

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[illegible] well and the other towards the anode appeared regularly in CIE tests of the various antigens with homologous or heterologous antisera in the reference antibody gel

Crossed line and Rocket line Immunoelectrophoresis

Comparative CIE tests of homologous and heterologous GC antigen antibody combinations were unrewarding because of the complexity of the precipitin lines demonstrated C LIE and R LIE tests with antigen containing intermediate agar gel as described by *Axelsen et al* (3) were therefore performed since these techniques permit the demonstration of strain-specific antigens in complex systems The overall results of these tests are summarized in Table 1 The homologous GC strains formed a specific precipitin line with their homologous antibodies when sonicated cells of one

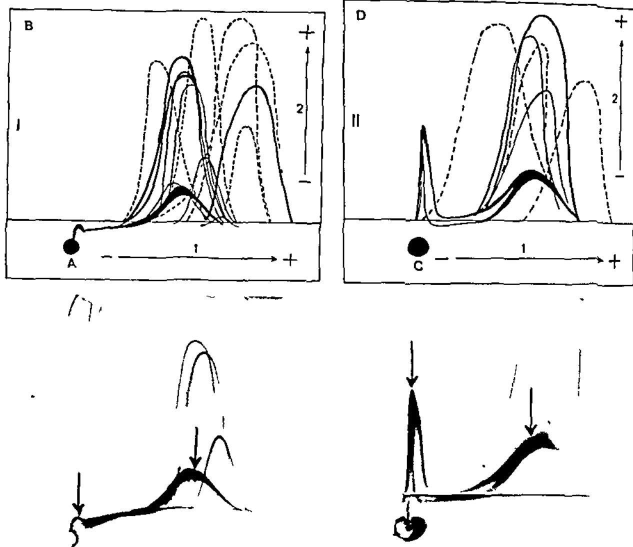


Fig 1 and 2 Schematic drawings (I & II) and photos of crossed immunoelectrophoresis tests of GC antigens with homologous anti GC antiserum The round basins A and C were filled with antigens of GC A and GC D respectively before the first electrophoretic run (arrow 1) the rectangular areas B and D with agarose containing antiGC A and anti GC D antiserum before the second electrophoretic run (arrow 2) The heavy and regularly appearing precipitin lines with two peaks (see text) are marked with → Dashed lines on the drawings indicate precipitin lines which were faint but clearly visible on the original plates

of the other three heterologous strains were included in the intermediate agar gel Figure 3 illustrates the specific precipitin line formed in such a C-LIE test It had two peaks one close to the antigen well and the other towards the anode The shape and position of this band corresponded to the one regularly formed in the CIE tests (Figures 1 & 2) but was less heavy Figure 4 demonstrates how these strain specific antigens appeared as rocket lines in R LIE tests which allowed examination of antigens from six or seven strains at a time in one microplate Figures 3 and 4 also show that the common antigens between the examined strains in the antigen wells and between those in the intermediate agar gel formed crossed lines or fused rocket lines with the reference antibodies It will be seen from Table I that there was an antigenic relationship between strains *A* and *B* when antigens of strain *D* were included in the intermediate agar gel

Effect of Heat Treatment

The strain specific antigens demonstrated in C-LIE and R-LIE tests with heterologous GC antigen in the intermediate agar gel also appeared after treatment with heat at 100° C for 1 h, but not after heating at 120° C

TABLE 1 *Results of Crossed line and Rocket line Immunoelectrophoresis (C LIE & R LIE) with Homologous and Heterologous Antigen antibody Combinations and with Homologous and Heterologous Antigens in the Intermediate Agar Gel*

Anti GC antisera used in C LIE and R LIE	Antigens of sonicated GC cells in intermediate gel	Specific precipitin or »rocket« lines formed in C LIE and R LIE tests with			
		GC A	GC B	GC-C	GC D
Anti A	A				
	B	pl/rl[1]			
	C	pl/rl			
	D	pl/rl	pl/rl		
Anti B	A		pl/rl		
	B				
	C		pl/rl		
	D	pl/rl	pl/rl		
Anti C	A			pl/rl	
	B			pl/rl	
	C				
	D	–/(rl)[2]	–(rl)	pl/rl	
Anti D	A				pl/rl
	B				pl/rl
	C				pl/rl
	D				

[1] pl specific precipitin line formed in C LIE (See Fig 3)
rl specific rocket line formed in R LIE (See Fig 4)
[2] (rl) rocket line formed was faint but clearly visible

C LIE Tests with Intermediate Agar Gel Containing Specific Antibodies

C LIE ...

line with two peaks was formed which had a posit on and nat e ...

gel

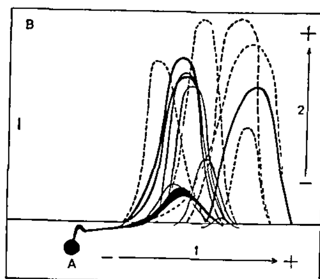
Co agglutination and Indirect Immunofluorescence of whole GC Cells

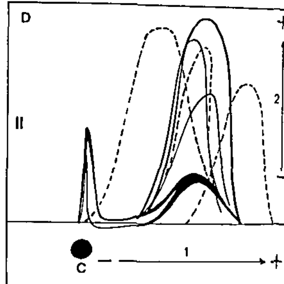
COA and indirect IFL tests were performed with whole untreated GC cells with unabsorbed anti GC antisera as used in the ...

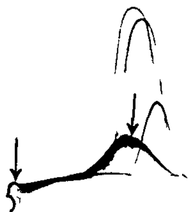
... ogous organisms varied from 1 1–1 20 for COA and from 1 80–1 1280 for IFL. Anti GC D differed from the other antisera and gave significantly lower titres with heterologous organisms in ...

... only of those from the rabbits first immunized with GC organisms in complete Freund s adjuvant

Since unabsorbed antisera did not discriminate between the GC test strains COA and indirect IFL tests were performed with anti GC antibodies absorbed with homologous or heterologous GC organisms The results of these tests are summarized in Tables 2 and 3 The tests were negative after absorption of the antisera with their homologous organisms Absorptions with heterologous organisms abolished the reactions with the organisms of the absorbing strains while positive reactions were consistently obtained with the homologous ones Strains *A* and *B* crossreacted after absorption of their antisera with strains *C* and *D* and they also reacted with anti *C* absorbed with *D* It can be seen from Tables 3 and 4 that the COA reactions here







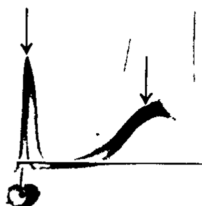


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of the other three heterologous strains were included in the intermediate agar gel Figure 3 illustrates the specific precipitin line formed in such a C-LIE test It had two peaks one close to the antigen well and the other towards the anode The shape and position of this band corresponded to the one regularly formed in the CIE tests (Figures 1 & 2) but was less heavy Figure 4 demonstrates how these strain specific antigens appeared as rocket lines in R-LIE tests which allowed examination of antigens from six or seven strains at a time in one microplate Figures 3 and 4 also show that the common antigens between the examined strains in the antigen wells and between those in the intermediate agar gel formed crossed lines or fused rocket lines with the reference antibodies It will be seen from Table 1 that there was an antigenic relationship between strains *A* and *B* when antigens of strain *D* were included in the intermediate agar gel

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		GC A	GC B	GC-C	GC D
Anti A	A				
	B	pl/rl*)			
	C	pl/rl			
	D	pl/rl	pl/rl		
Anti B	A		pl/rl		
	B				
	C		pl/rl		
	D	pl/rl	pl/rl		
Anti C	A			pl/rl	
	B			pl/rl	
	C				
	D	–/(rl)**	–(rl)	pl/rl	
Anti D	A				pl/rl
	B				pl/rl
	C				pl/rl
	D				

*) pl specific precipitin line formed in C LIE (See Fig 3)
rl specific »rocket« line formed in R LIE (See Fig 4)
**) (rl) »rocket« line formed was faint but clearly visible

C LIE Tests with Intermediate Agar Gel Containing Specific Antibodies

C LIE tests were also carried out with intermediate agar gel containing holomogous GC antibodies absorbed with heterologous GC strains A precipitin line with two peaks was formed which had a position and pattern corresponding to the strain specific line (see Fig 4) demonstrated in C LIE tests with heterologous antigen in the intermediate agar gel

Co agglutination and Indirect Immunofluorescence of whole GC Cells

COA and indirect IFL tests were performed with whole untreated GC cells with unabsorbed anti GC ant sera

tests

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1 5-1 ... ution of 0 1 ml of antiserum to make 10 ml reagent staphylococci) and indirect IFL titres from 1 640–1 1280 The corresponding titres with heterologous organisms varied from 1 1–1 20 for COA and from 1 80–1 1280 for IFL Anti GC D differed from the other antisera and gave signifi

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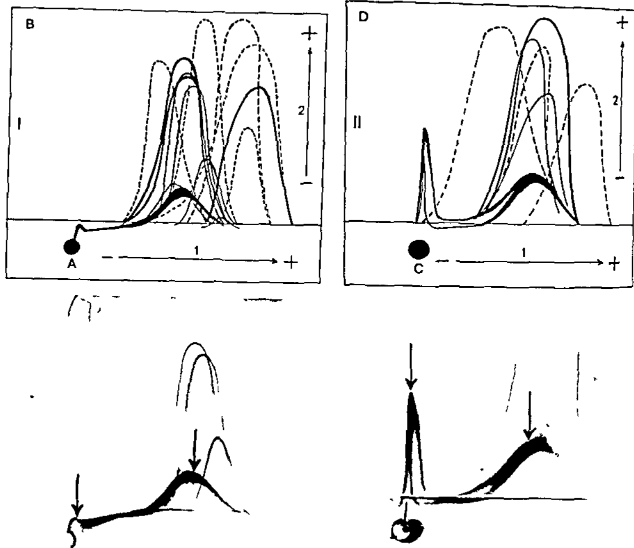


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TABLE 2 *Co-agglutination Reactions of GC Organisms with Reagent Staphylococci Coated with Anti-GC Antibodies Absorbed with Homologous or Heterologous Strains of* Neisseria gonorrhoeae. *The Final Dilution of Absorbed Antisera Used for Coating of Staphylococci was 1:2. Reactions Graded as Described in Materials and Methods*

Reagent staphylococci coated with absorbed anti-GC antibodies	Absorbing GC strain	Co-agglutination reactions obtained with			
		GC A	GC B	GC-C	GC-D
Anti-A	A				
	B	+ +			
	C	+ + +	+ +		
	D	+ + +	+ +		
Anti-B	A		+ +		
	B				
	C	(occ[1]) + +)	+ + +		
	D	+ +	+ + +	(occ + +)	
Anti-C	A			+ + +	
	B			+ + +	
	C				
	D	+ +	+ +	+ + +	
Anti-D	A				+ + +
	B				+ +
	C				+ +
	D				

[1]) occ: occasionally tests gave + + reactions

TABLE 3 *Results of Indirect IFL Tests of GC Organisms with Anti-GC Antibodies Absorbed with Homologous or Heterologous Strains of* Neisseria gonorrhoeae. *Titres > 1:10 are Indicated*

Absorbed anti-GC antisera used in indirect IFL	Absorbing GC strain	Indirect IFL titres (reciprocal) obtained with			
		GC A	GC B	GC-C	GC D
Anti-A	A				
	B	*160*			
	C	*160*	40		
	D	*160*	40		
Anti-B	A		*160*		
	B				
	C	160	*640*		
	D	160	*640*	160	
Anti-C	A			*160*	
	B			*160*	
	C				
	D	160	160	*160*	
Anti-D	A				
	B				*160*
	C				*160*
	D				*160*

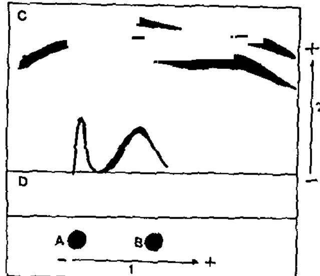


Fig 3 Schematic drawing and photo of crossed line immunoelectrophoresis with antigen containing intermediate agar gel Basins A and B were filled with antigens of GC D and GC A respectively before the first electrophoretic run (arrow 1) the rectangular area C with agarose containing anti GC D antiserum and area D (intermediate Gel) with agarose containing GC A before the second run (arrow 2) The antigens of GC A in the intermediate gel have formed crossed lines (marked with →) in common with those of GC D Antigens of GC D in basin A have formed a specific precipitin band with two peaks The antigens of GC A in basin B formed no precipitin band (see Table 1)

Fig 4 Photo of rocket line immunoelectrophoresis Basins 1–6 were filled with GC D (1) GC A (2) GC B (3) GC C (4) GC A (5) and GC D (6) the intermediate agar gel containing GC C antigen and the reference antibody gel containing anti GC A before the electrophoretic run Antigens in the intermediate gel and in the basins have formed crossed lines with peaks representing common antigen Antigens in basins 2 & 5 (GC A) have formed specific rocket lines indicated by →

were weaker or variable, and the indirect IFL titres significantly lower

A comparison of the results given in Tables 2 and 3 with those of Table 1 shows that COA and indirect IFL tests of whole cells with absorbed antisera were congruent with the results of C LIE and R LIE tests with antigen containing intermediate agar gel

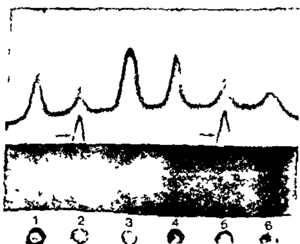
COA and Indirect IFL of GC Cells Treated by Heat

GC cells treated by heat at 100° C for 1 h gave the same reactions as the untreated cells with the reagent staphyloccocci coated with absorbed antibodies The reactions with the heated GC cells were generally even more clearcut, and + + reactions with untreated cells were often read as + + + COA tests of GC cells treated by heat at 120° C for 1 h were regularly negative with the specific reagent staphylococci

Indirect IFL tests with absorbed antisera gave the same results with GC organisms treated by heat at 100° C for 1 h as with the untreated However cells treated by heat at 120° C appeared differently and a typical peripheral staining could only occasionally be seen

COA and indirect IFL with GC organisms of colony morphology types T3/T4

Indirect IFL tests with unabsorbed or absorbed antisera were similar with GC cells of colony morphology types T3/T4 as with those of T2 In this respect the COA reactions differed from those of IFL As can be seen from Table 4 the reactions with reagent staphylococci coated with unabsorbed or absorbed antibodies were read as weak or negative with untreated organisms of T3/T4 colonies in contrast to + + + reactions with those of T2 colonies The table shows however that after



the COA tests but they agglutinated after being heated at 100° C for 1 h *Wilson* (30) also described inagglutinable GC organisms which were not however referred to colony morphology types at that time Noteworthy are also the COA reactions of strains A and C with absorbed anti B antibodies obtained occasionally (Table 2) Further studies have shown that this may be due to the occurrence of colour/opacity colonial variants of T2 organisms described by *Swanson* (26) (*Danielsson & Sandstrom* in preparation)

An indirect IFL technique the so-called micro-immunofluorescence test was used recently by *Wang et al* (29) for serological classification of GC strains With the use of cross absorbed anti GC antisera we found the standard indirect IFL technique useful for serological differentiation of GC strains The results agreed with the strain specific antigens demonstrated by C LIE R LIE and COA Like *Wang et al* (29) we found the results to be dependent on which GC strains were used for absorbing the antisera Our findings like those of *O Reilly et al* (21) and *Wang et al* (29) indicated that some GC strains have a broader antigenic spectrum than others

Recently *Smyth et al* (24) were able to differentiate GC membrane antigens from those of protoplasmic origin by the use of CIE which has a higher resolution than the Ouchterlony method for studying precipitating antigen antibody systems (3) To our knowledge the present work is the first report of strain specific GC antigens with C LIE and R LIE using the antigen intermediate agar gel technique The R LIE was found to be useful for comparative studies of antigens from several GC strains at a time This method was also somewhat more sensitive than C LIE for detecting cross reacting antigens These immunoelectrophoresis techniques in combination with COA and IFL methods have a great potential use for serological studies of antigenic fractions and whole cells of *Neisseria gonorrhoeae* organisms treated in various ways by physical or chemical means

The precipitin lines demonstrated by C LIE and R LIE to represent strain specific antigens indicated complexity It is of interest to note however that their basic pattern was similar for the four GC strains studied Antibodies reacting with these antigens were also demonstrated regularly in the sera of rabbits hyperimmunized with antigen in complete Freund s adjuvant or by the (iv) route only This was in contrast to the other precipitin lines the appearance of which was dependent on the antiserum used Our findings indicate therefore that the strain specific antigens were highly immunogenic

This work was supported by grants from the *Swedish Medical Research Council* (Project No 4778) and from the World Health Organization We are indebted to Mrs *G Malmros* and Mrs *M Jurstrand* for technical assistance and to Miss *E Gustavsson* for typing the manuscript

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TABLE 4 *Co-agglutination Reactions of Untreated and Heat treated GC Organisms of Colony Morphology Types T2 and T3/T4 with reagent Staphylococci Coated with Unobsorbed or Absorbed Anti-GC Antibodies*

Test strain and colony morphology	Treatment of cells	Anti GC-A		Anti GC C	
		Not abs	Abs with GC-B	Not abs	Abs with GC A
GC-A, T2	None	+ + +	+ + +	+ + +	–
	100° C	+ + +	+ + +	+ + +	–
GC-A, T3/T4	None	±	–	±	–
	100° C	+ + +	+ + +	+ + +	–
GC-C, T2	None	+ + +	–	+ + +	+ + +
	100° C	+ + +	–	+ + +	+ + +
GC-C, T3/T4	None	±	–	±	–
	100° C	+ + +	–	+ + +	+ + +

heating of the T3/T4 cells at 100° C for 1 h, the reactions were congruent with those of the untreated or heat-treated T2 cells

Relationship of Strain-specific Antigens Demonstrated in C-LIE, R-LIE and COA

Experiments were carried out to study the relationship of the strain specific antigen(s) demonstrated in C-LIE and R LIE tests with sonicated antigens to those shown by COA tests with whole GC organisms

Reagent staphylococci coated with absorbed anti GC-A antibodies (absorbed with strain C and reactive only with A, Table 2) were used as immunosorbent and were mixed with sonicated cells of strain A A control test was performed with uncoated staphylococci The mixtures were incubated at 37° C for 2 h, after which the staphylococci were removed by centrifugation Both the immuno sorbed GC-A antigen and the control antigen were then tested in C-LIE and R-LIE with strain C antigen in the intermediate gel and with anti GC-A in the reference antibody gel The strain-specific line was formed with the control antigen but not with the immunosorbed antigen A COA test with specific reagent staphylococci and sonicated antigen was positive before immunosorption but negative afterwards Corresponding immunosorption experiments with the other three GC antigen-antibody systems gave similar results

DISCUSSION

The Ouchterlony method and direct and indirect IFL techniques were used in previous studies to demonstrate antigenic similarity and diversity among strains of *Neisseria gonorrhoeae* (8, 12, 14, 29) These observations were confirmed in the present investigation, and both common and strain specific antigens were demonstrated by two immunoelectrophoresis methods, C LIE and R-LIE of disrupted organisms, and by indirect IFL of whole cells Corresponding results were also arrived at by the COA technique, which we found to be sensitive rapid and simple Moreover, this technique needed no special equipment

The inherent autoagglutination of both virulent and avirulent GC cells (16, 25) have made serological studies of these organisms by classical agglutination methods unrewarding (27, 30) The COA method offers an advantage from this point of view, since it is not influenced by autoagglutinating bacteria (6, 10, 17) It can also be used to study soluble antigenic fractions or antigens of disrupted organisms as shown in the present work and by others (17 20) COA studies of whole cells of pneumococci (17), streptococci (6) mycobacteria (15) and meningococci (20) have shown that the specific antibodies coated on protein A-containing staphylococci react with surface antigens expressing serogroup or serotype specificities It may be assumed therefore that the strain specific antigens demonstrated by the COA method and by the other two immunological techniques used were probably constituents of the outer membranes Our results indicated that they were resistant to heat at 100° C but not at 120° C It is worth noting that the strain-specific antigens demonstrated by C-LIE and R-LIE could be removed by immunosorption with reagent staphylococci coated with the corresponding strain-specific antibodies

It is of interest that untreated gonococci of colony morphology types T3 and T4 were inagglutinable in

the COA tests but they agglutinated after being heated at 100° C for 1 h *Wilson* (30) also described inagglutinable GC organisms which were not however referred to colony morphology types at that time Noteworthy are also the COA reactions of strains A and C with absorbed anti B antibodies obtained occasionally (Table 2) Further studies have shown that this may be due to the occurrence of colour/opacity colonial variants of T2 organisms described by *Swanson* (26) (*Danielsson & Sandstrom* in preparation)

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EXPERIMENTAL ACUTE SALPINGITIS IN GRIVET MONKEYS PROVOKED BY *CHLAMYDIA TRACHOMATIS*

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Ripa K T Møller B R Mårdh P A Freundt E A & Melsen F Experimental acute salpingitis in grivet monkeys provoked by *Chlamydia trachomatis* Acta path microbiol scand Sect B *87* 65–70 1979

Chlamydia trachomatis is a common cause of sexually transmitted diseases Recently it has been shown that chlamydiae are also responsible for complications to such lower genital tract infections In this study isolates of *C trachomatis* from the fallopian tubes of patients with acute salpingitis were inoculated direct into the fallopian tubes of two and through the cervical canal into the uterine cavity of one grivet monkey The experimental infections resulted in a self limited acute salpingitis in the three animals *C trachomatis* was recovered from the monkeys 2 and 3 weeks post inoculation As found at laparotomy the infected tubes were swollen and reddened and there was watery exudate in the abdominal ostia Microscopically cellular infiltrates – mainly lymphocytes – were seen in the mucosa muscularis and subserosa of the tubes Serologically a primary antibody response with an IgM to IgG conversion was found Salpingitis did not occur in a control monkey inoculated in the tubes with a medium lacking *Chlamydia* The histological changes in the fallopian tubes of the infected monkeys were reminiscent of those described as being characteristic of ›gonococcal‹ salpingitis in man The fulfilment of Koch s postulates in the animal model used adds to the earlier evidence that *C trachomatis* is capable of causing acute salpingitis in humans

Key words *Chlamydia trachomatis* salpingitis experimental infection

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Acute salpingitis generally a complication of sexually transmitted lower genital tract infection is a disease with an increasing incidence Chlamydiae gonococci mycoplasmas and strictly anaerobic bacteria have all been considered to be etiological agents of this disease (6 9 10)

Chlamydia trachomatis is now believed to be the most common cause of sexually transmitted diseases both in Europe and in the U S (1 14 15) A substantial proportion of all cases of acute salpingitis in our hospital catchment region are a result of *C trachomatis* infection as indicated by culture studies including isolations from the fallopian tubes (10)

Experimental genital infections with *C trachomatis* [illegible] infections of the fallopian tubes by mycoplasmas (11)

In studies on the role of chlamydiae in acute salpingitis we inoculated *C trachomatis* into the fallopian tubes or into the uterine cavity of grivet monkeys The monkeys were repeatedly laparotomized during 12 weeks after the infection The macroscopic lesions of the tubes were registered and biopsies were taken for histological studies The development of antibodies to *C trachomatis* are reported as are the results of culture studies

MATERIALS AND METHODS

Monkeys Four female grivet monkeys (*Cercopethecus aethiops*) which had been captured in East Africa were used They had been kept in quarantine for a minimum of 6 weeks before use in the experiments During the investigation period the monkeys were housed in individual cages in an isolated room They were fed with a commercial primate food supplemented with fresh fruit and water At the start of the experiments their weights were 2 0–2 3 kg

Chlamydial strains Two strains of *C trachomatis* immunotypes K and I which had been isolated from the fallopian tubes of two patients with acute salpingitis were used The strains had been isolated on cycloheximide treated McCoy cells (13) and passaged twice in the yolk sac of embryonated hens eggs After the second passage the yolk sac material was frozen to −80° C as a 20% suspension in sucrose phosphate buffer (2 SP) (7) containing 10 μg gentamicin and 2 5 μg amphotericin B per ml The two suspensions contained 2×10^5 inclusion forming units each of chlamydiae per ml A 1 100 dilution of the yolk sac specimens in phosphate buffered saline was used to infect the animals A yolk sac specimen from a non infected egg was treated in the same way and used to inoculate the control monkey

Sampling before inoculation Before the experiments were started swabs were collected at three consecutive occasions from the throat the cervix and the rectum The sampling was made when the animals were under general anaesthesia using ketamine chloride (Ketalar® 5 mg/kg i m) The samples were studied for the occurrence of *C trachomatis* and *Mycoplasma hominis* In addition cervical specimens were studied for *Trichomonas vaginalis*

Blood samples for antibody determinations leukocyte counting and determination of the erythrocyte sedimentation rate (ESR) were taken under general anaesthesia by puncture of the femoral artery in the inguinal region

The rectal temperature was recorded daily with a thermistor

Infection of the monkeys The animals were anaesthetized using 0 15 ml pheynylclidine hydrochloride (Sernylan® 20 mg/ml) 0 5 ml chlorpromazine (0 25 % solution) and 0 2 ml atropine (0 1 % solution) Surgery was performed under aseptic conditions as described elsewhere (11) Of the chlamydial suspension 0 2 ml (corresponding to approximately 200 inclusion forming units) was injected into the lumen through the wall of the lateral part of each fallopian tube using an 0 8 mm needle One monkey was inoculated with *C trachomatis* immunotype I and another with immunotype K The control animal was injected with the non infected yolk sac specimen in the same way One monkey was inoculated through the cervical canal into the uterine cavity with immunotype K

Assessment of lesions and collection of specimens Laparotomy was performed as described above on days 3 7 14 21 35 56 70 and 84 post infection (p i) On all days mentioned biopsies were taken from the tubes and the parametria by means of a 2 mm surgical ear forceps

On each day of laparotomy specimens were collected with swabs from the cul de sac the abdominal ostium of the fallopian tubes and from the cervix The specimens were studied for the presence of chlamydiae mycoplasmas and bacteria From the monkey inoculated into the uterine cavity biopsy specimens from the tubes were cultured for chlamydiae

Blood samples were taken from the femoral artery on the days of laparotomy and on day 28 p i

Culture studies *C trachomatis* was cultured using cycloheximide treated McCoy cells as described earlier (13) The culture technique for *M hominis* has been described in detail elsewhere (11) Cultures for bacteria were made on blood haematin and lactulose bromthymol blue agar plates and in brain heart infusion broth

Serological studies The presence of serum antibodies to *C trachomatis* was studied using a micro immunofluorescence test (16) Fluorescein isothiocyanate conjugated rabbit anti human anti IgG and anti IgM globulin (Dakopatts A/S Copenhagen Denmark) were used The monkey sera were titrated in twofold dilutions The lowest dilution used was 1 8

Histological studies The tissue specimens were fixed in 10% formalin and processed by staining with hematoxylin and eosin

RESULTS

General condition leukocyte count ESR and body temperature During the experimental period the general condition of the four monkeys remained unimpaired No rise in rectal temperature was observed The monkeys gained 0 1–0 2 kg in weight during the investigation period of 84 days

The ESR was moderately elevated on days 3–14 in all three monkeys inoculated with *C trachomatis* viz from 1 to 13 mm from 2 to 8 mm and from 1–24 mm respectively The leukocyte count in the same monkeys increased from 9300 to 13800 from 6900 to 8000 and from 6500 to 7600 per mm³ from days 7–14 In the control monkey ESR was 0 to 4 mm There was no change in the leukocyte counts

Gross lesions No signs of inflammation of the fallopian tubes were seen on day 3 p i Four days later the tubes were swollen and reddened and exudate appeared in the abdominal ostia of the tubes – findings which were fairly unchanged on day 14 and 21 p i Thereafter the gross lesions regressed On day 35 the tubes looked virtually normal On days 7–35 p i a serous watery exudate was found in the abdominal ostia of the tubes and in the cul-de sac Purulent exudate never occurred The parametria the ovaries and the uterus were macroscopically invariably normal throughout the experimental period Cystic structures did not develop

In the control monkey no inflammatory changes were found in the tubes and parametria



Figures 1–4 are histological sections of monkey uterine tubes stained with hematoxylin and eosin Figures 1–3 are from a monkey inoculated into the fallopian tube with 200 inclusion forming units of *C trachomatis* immunotype K Figure 4 is from a monkey inoculated through the cervical canal into the uterine cavity with 200 inclusion forming units of *C trachomatis* immunotype K

Fig 1 Section of a normal tube at day of infection × 40

Fig 2 Early inflammatory reaction involving the epithelium and subepithelial connective tissue Exudate in the tubal lumen Day 7 post infection × 100

Fig 3 Pronounced inflammatory infiltration of subepithelial tissue and partly of the muscular layer Day 14 post infection × 60

Fig 4 Pronounced inflammatory infiltrate in the subepithelial tissue and partly in the epithelium Day 3 post infection × 100

Histological findings Pronounced inflammatory changes of the fallopian tubes were found in the infected monkeys Already on day 3 p i there was an infiltration of the subepithelial tissue partly involving the epithelium with abundant lymphocytes and some polymorphonuclear leukocytes The cellular infiltration extended into the muscular layers The tubal epithelium was morphologically intact and no exudate was seen in the tubal lumen

On day 7 p i a marked inflammatory infiltrate involved the mucosal epithelium which was injured in some areas The mucosal folds were oedematous and infiltrated with lymphocytes The inflammatory reaction involved the subepithelial connective tissue In the lumen an exudate containing lymphocytes and small clusters of desquamated epithelium was seen The histological findings during the following 3–4 weeks were substantially unchanged On day 35 a moderate cellular infiltration was found in the epithelium and in the mucosa The epithelial lining of the tubes was then practically intact An exudate was still found in the lumen The inflammation declined during the subsequent weeks but on day 55 p i a small number of inflammatory cells were st ll …

In the fallopian tubes of the control monkey only

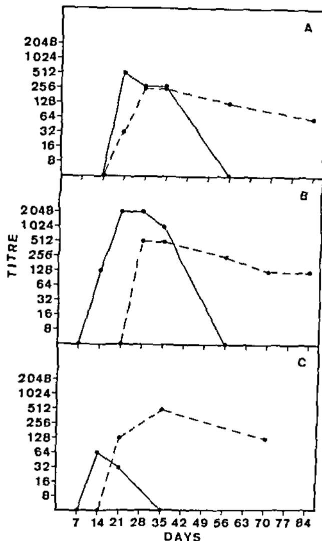


Fig 5 Reciprocal IgM (●—●) and IgG (● - - - ●) antibody titres to *C trachomatis* in three grivet monkeys inoculated with immunotype I (A) and K (B) into the uterine tubes and immunotype K (C) into the uterine cavity

slight inflammatory changes were found in the subserosa The muscular layers the tubal mucosa and epithelium showed no signs of inflammation throughout the experimental period There was no exudate in the lumen

The parametria of all four monkeys studied presented grossly no signs of inflammation The histological examination revealed only a moderate inflammatory infiltration of the subserosa No oedema or hyperaemia involved the tissues studied

Culture studies Before infection no chlamydiae or mycoplasmas could be isolated from the throat the cervix or the rectum of any of the four monkeys *C trachomatis* was isolated from cervical specimens from the two monkeys inoculated direct into the fallopian tubes but only on days 14 and 21 p i Chlamydiae could not be isolated from the abdominal ostia of the tubes or from the cul de sac from these two animals From the monkey inoculated into the uterine cavity *C trachomatis* was recovered on day 21 p i from tubal biopsy and cul-de sac specimens The strains recovered from each animal were of the same immunotype of *C trachomatis* as that used to infect the animal On no occasion chlamydiae were detected in the control monkey Neither mycoplasmas nor bacteria were recovered from the tubes or the peritoneal cavity of any of the four monkeys

Antibody determinations No IgM or IgG antibodies to *C trachomatis* were detected in serum samples taken before infection IgM antibodies reacting with pool II in the test system (containing antigens prepared from *C trachomatis* immunotypes D E F G H I and K) were detected on day 14 p i in the two monkeys infected with immunotype K and on day 21 p i in the animal infected with immunotype I The titres were 1 64 in the animal inoculated into the uterine cavity and 1 2048 and 1 512 in the other two The IgM antibodies were non detectable after 5–8 weeks p i IgG antibodies reacting with the same pool of antigens were detected on days 21–28 the highest titres were found during the 4th and the 5th week viz 1 512 and 1 256 and were still detectable at the end of the experimental period 10–12 weeks p i (Fig 5)

No IgM or IgG antibodies to *C trachomatis* were detectable in the serum samples from the control monkey

DISCUSSION

During the last decade substantial evidence has accumulated that *C trachomatis* is a major etiological agent of sexually transmitted diseases (1 14 15) Recently it has been demonstrated that the organism is an important cause of complications in such infections in both the male and the female i e acute epididymitis (3) and salpingitis (10)

In an earlier study (10) we isolated *C trachomatis* from 19/53 patients with acute salpingitis and from the fallopian tubes of 6/20 of these patients We also found serological evidence of a recent or active chlamydial infection in 62% of a series of 143 female patients living in southern Sweden who had laparoscopically verified acute salpingitis (*J D Treharne K T Ripa P A Mardh L Svensson L Westrom and S Darougar* Br J Vener Dis in *press*)

Hitherto no natural infections with *C trachomatis* have been reported in non human primates None of the four grivet monkeys included in our study (and none of 24 other such monkeys studied

by us) harboured chlamydiae in the lower genital tract the rectum or the pharynx

Experimental infection of the lower genital tract and the eyes of monkeys by means of strains of *C trachomatis* isolated from humans have been found to produce clinical infections (2 4 5)

The present study shows that it is possible to elicit acute salpingitis in grivet monkeys by inoculation of chlamydiae direct into fallopian tubes or into the uterine cavity Histological signs of inflammation in the tubes were found between 3 days and 8 weeks after the inoculation The endotubal infection thus caused a self limiting salpingitis The antibody response was that of a primary infection with an IgM to IgG antibody conversion The IgM titres reached a height generally not seen in infections with *C trachomatis* in man

Re isolation of chlamydiae from the two monkeys inoculated into the tubal lumina was only successful from cervical specimens and only on two sampling occasions Apparently the infection had spread in the opposite direction proposed to that occurring in natural infections with chlamydiae in the female Sampling from the fallopian tubes of these monkeys was done by swabbing the fimbriated ends of the tubes since the small size of the tubes do not allow endotubal sampling This might explain the negative culture results On the other hand from the monkey inoculated into the uterine cavity *C trachomatis* could be isolated from the tubes 3 weeks p i Tubal biopsy specimens were used for cultures from the aminal

The histological changes in the tubes of the grivet monkeys resembled those described in cases of ›gonococcal‹ salpingitis in man (12) The epithelium of the tubes was affected and the lumen contained exudate which was also seen in the abdominal ostiae of the tubes

Gonococci are recovered from the fallopian tubes in only a fraction of patients with cervical gonorrhoea and acute salpingitis even when examined in the acute stage (9) Salpingitis cases have often been classified as ›gonococcal‹ in spite of the lack of bacteriological studies on samples from the site of infection viz the uterine tubes

In ›non gonococcal salpingitis described in text books as occurring as a postpartum or postabortive complication or as a complication of surgical proced [illegible]

[illegible] is believed to gain entrance to the internal female genital organs through lesions in the cervical mucosa or the endometrial epithelium and spreads thence to the parametria broad ligaments and later to the tubes via blood vessels and lymphatics In a recent investigation (11) the type of infection mentioned has been shown to develop in grivet monkeys after inoculation of *Mycoplasma hominis* in the same way as used in our study

Double infections with *N gonorrhoeae* and *C trachomatis* commonly occur in the cervix (8) The histological changes in the tubes caused either by gonococci or chlamydiae have many features in common as discussed above Both types of infections seem to be spread canalicularly from the cervical canal to the endosalpinx involving the epithelium and producing an inflammatory exudate in the lumen

We have recently examined an extirpated fallopian tube from a patient with acute pyosalpinx from which *C trachomatis* but no other organisms including gonococci could be recovered Nor did cervical cultures reveal any other organisms possibly causing the salpingitis (to be published) The mucosal folds of the tube were oedematous and infiltrated with polymorphonuclear and mononuclear leukocytes There were marked reactive changes in the tubal epithelium The inflammatory infiltrate and the oedema involved the tubal wall and the serosa The pathological changes in this patient were the same as those described to be characteristic of »gonococcal« salpingitis (12) and also similar to those found in the tubes of the three experimentally infected monkeys in this study

Thus the experimental acute salpingitis produced in monkeys and the findings in the patient with chlamydial pyosalpinx suggest that salpingitis caused by *C trachomatis* leads to histological changes in the tubes which are very similar to those described as occurring in »gonococcal« salpingitis while *M hominis* infections (11) produces changes similar to those found in patients with »non gonococcal« salpingitis

The isolation of C *trachomatis* from the fallopian tubes of patients with acute salpingitis the detection of specific antibodies in these patients and the fulfilment of Koch s postulates in the animal model used indicate that C *trachomatis* can cause acute salpingitis

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The serotyping of the chlamydial strains used was kindly performed by *J Treharne* Insitute of Ophthalmology University of London U K

This work was s[illegible]

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Acta path microbiol scand Sect. B 87 71–73 1979

BRIEF REPORT

NEUTRALIZATION OF AGGREGATED STRAINS OF ENTEROVIRUS 71 AND ECHOVIRUS TYPE 4 IN RD AND VERO OR GMK AH1 CELLS

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Zeipel G von Neutralization of aggregated strains of enterovirus 71 and echovirus type 4 in RD and Vero or GMK AH1 cells Acta path microbiol scand Sect. B 87 71–73 1979

Strains of enterovirus 71 and echovirus type 4 containing aggregates which were poorly neutralized by antibody in cell lines of green monkey kidney origin such as Vero and GMK AH1 were as easily neutralized as non aggregated strains when grown in human RD cells The explanation for this may be a lesser degree of aggregation of viral materials originating from RD cells and/or the use of lower doses of virus for neutralization tests in such cells The latter was a consequence of a higher sensitivity of RD cells to minimal amounts of virus than that shown by Vero and GMK AH1 cells Thus virus grown in RD Vero or GMK AH1 cells reached a titre 10 to 100 times higher in RD cells than in the other cell lines

Key words Cell line enteroviruses neutralization tests viral antigens

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Enterovirus 71 was first isolated from cases of aseptic meningitis in Stockholm in 1967 (1) and has since given rise

an epidemic of severe polio like disease in Bulgaria (2)

The typing of enterovirus 71 has raised some difficulties in several countries including Sweden (1) U S A (7) and Australia (6) since many strains have been poorly neutralizable This is apparently due to their high content of viral aggregates which prevents neutralizing antibodies from reaching hidden infectious particles Effective neutralization is achieved if the aggregates are removed by filtration (9) prior to the neutralization test or occasionally if they are dissolved by the addition of sodium deoxycholate (4) As shown in the present study however such procedures are unnecessary if the neutralization tests are performed in RD cells

Materials and Methods

Roller tube cultures of Vero and RD (8) cells were maintained in a medium composed of equal parts of Eagle s Basal Medium and Medium 199 with 20 per cent tryptose phosphate broth and 2 per cent inactivated foetal calf serum For GMK AH1 cells serum was omitted from the medium

without prefiltration (1)

Echovirus type 4 The prototype strain Pesascek non neutralizable in GMK AH1 cultures without prefiltration and the directly neutralizable strain Du Toit were used (9)

Hyperimmune sera Monkey serum against strain BG 258 guinea pig sera against S 52500 and Du Toit and horse serum against Pesascek were used

Results

Pesascek were neutralized only in RD cells Strains S 52343 and S 6041 behaved like the latter strains The virus used in these neutralization tests were

TABLE 1 *Neutralization of Enterovirus 71 and Echovirus 4 in Cell Cultures of RD Vero and GMK AH1*

Virus strain	TCD_{50} in tests	Antiserum against	Titre of serum in RD	Titre of serum in Vero* GMK**
BG 258	60–160	BG 258	9600[a]	12000*
»	»	S 52500	12000	6000*
S 52500	250–400	S 52500	6800	30*
»	»	BG 258	3300	30*
Echo 4 Du Toit	160–250	E 4 Du T	2250	860**
Echo 4 Pesascek	60	E 4 Du T	1560	<30**
»	250	E 4 Pes	2200	<20**

a Titre of serum given as the inverted value of the serum dilution per 0 05 ml

grown in the same kind of cells as those used for the particular test Serum titres were taken 6 to 7 days after the break through of the virus dose in the tests

Other experiments showed that Pesascek virus originating from GMK AH1 cells was also directly neutralizable if tested in RD cells whereas virus grown in RD cells was less well neutralized when tested in GMK AH1 cells though clearly better than the apparently n aggregated virus grown in GMK AH1 cells

Further studies revealed that RD cells were n susceptible to minimal amounts of virus of the str tested than Vero and GMK AH1 cells As show Table 2 virus grown in RD Vero and GMK A cultures reached a titre 10 to 100 times higher in cells than in the other cell lines when inoculated from same dilution series Thus in reality higher dose virus containing more aggregates were used for neut zation tests with 100 TCD_{50} of virus in Vero and G AH1 cells than was indicated by the cytopathic effe these cells This would contribute to the poor neutral tion of aggregated strains in Vero and GMK AH1 c

All twelve strains of enterovirus 71 tested h without adaptation given a complete cytopathic effe RD cells in which so far also two new such strains h been isolated from vesicle fluids Work is in progres test the suitability of RD cells for the typing aggregated strains of enterovirus types other than th referred to above

TABLE 2 *Titres of Enterovirus 71 and Echovirus Type 4 in Tube Cultures of RD and Vero or GMK Cells*

Virus strain	Last passages in cell lines	Titre[b] in cells of RD	Titre[b] in cells of Vero* GMK**
BG 258	2 in Vero	6 5[c]	5 5*
»	2 in RD	6 7	6 1*
S 52500[a]	4 in Vero	7 5	5 3*
›	2 in RD	7 1	5 9*
S 52343[a]	2 in Vero	6 9	5 1*
›	2 in RD	7 7	5 5*
S 6041[a]	3 in Vero	6 3	4 3*
»	2 in RD	6 9	5 1*
Echo 4 Pesascek	2 in GMK AH1	8 1	6 7**
	4 in RD	7 9	6 7**
Echo 4 Du Toit	3 in GMK AH1	7 7	7 5**
	4 in RD	8 5	7 5**

a Aggregated strain of enterovirus 71
b The same dilution series of each material was tested simultaneously in RD and/or Vero/GMK AH1
c Log_{10} mean tissue culture dose per 0 1 ml

A Summary of this work was presented at International Congress of Infectious and Paras Diseases Varna Bulgaria October 1978

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Biol Med *148* 203–207 1975 – 4 *Gwaltney J M & Calhoun A M* Appl Microbiol *20* 390–392 1970 – 5 *Hagiwara A Tagaya I & Yoneyma T* Intervirology 9 60–63 1978 – 6 *Kennett M L Birch C J Lewis F A Yung A P Locarnini S A & Gust I D* Bull Wld Hlth Org *51* 609–615 1974 – 7 *Schmidt N J Lennette E H & Ho H H* J Inf Dis *129* 304–309 1974 – 8 *Schmidt N J Ho H H & Lennette E H* J Clin Microbiol *2* 183–185 1975 – 9 *Wallis C & Melnick J L* Virology *1* 478–488 1967

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All twelve strains of enterovirus 71 tested have without adaptation given a complete cytopathic effect in RD cells in which so far also two new such strains have been isolated from vesicle fluids Work is in progress to test the suitability of RD cells for the typing of aggregated strains of enterovirus types other than those referred to above

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Virus strain	Last passages in cell lines	Titre[b] in cells of RD	Titre[b] in cells of Vero* GMK**
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S 52500[a]	4 in Vero	7 5	5 3*
»	2 in RD	7 1	5 9*
S 52343[a]	2 in Vero	6 9	5 1*
»	2 in RD	7 7	5 5*
S 6041[a]	3 in Vero	6 3	4 3*
»	2 in RD	6 9	5 1*
Echo 4 Pesascek	2 in GMK AH1	8 1	6 7**
	4 in RD	7 9	6 7**
Echo 4 Du Toit	3 in GMK AH1	7 7	7 5**
	4 in RD	8 5	7 5**

[a] Aggregated strain of enterovirus 71
[b] The same dilution series of each material was tested simultaneously in RD and/or Vero/GMK AH1
[c] Log_{10} mean tissue culture dose per 0 1 ml

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BRIEF REPORT

TRISODIUM PHOSPHONOFORMATE INHIBITS HEPATITIS B DANE PARTICLE DNA POLYMERASE

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Nordenfelt, E Helgstrand E & Öberg B Trisodium phosphonoformate inhibits hepatitis B Dane particle DNA polymerase Acta path microbiol scand Sect B *87* 75–76 1979

Evidence available indicates that the so-called Dane particles are the hepatitis virus A DNA polymerase is associated with the core of these particles The probability that this is the viral DNA polymerase offers the possibility of preventing hepatitis B multiplication by selective inhibition of this enzyme This investigation reports that trisodium phosphonoformate (PFA) at low concentrations but not phosphonoacetate acid (PAA) inhibits Dane particle associated DNA polymerase

Key words Hepatitis B DNA polymerase inhibitor phosphonoformic acid (PFA)

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Hepatitis B virus (HBV) has yet not been isolated and cultured *in vitro* However all evidence available indicates that the so-called Dane particles are the HBV (*Dane et al* 1970) They are about 45 nm in diameter and have been

found to be associated with the core (*Hirschman et al* 1971 *Kaplan et al* 1973) The probability that this is the viral DNA polymerase (*Krugman et al* 1974) offers the possibility of preventing hepatitis B multiplication by selective inhibition of the Dane particle DNA polymerase and this enzyme has recently been utilized to select potential therapeutic agents for hepatitis B infection (*Hirschman & Garfinkel* 1978)

We now report that trisodium phosphonoformate (PFA) at low concentrations inhibits Dane particle DNA polymerase

In a screening programme utilizing viral enzymes PFA was found recently to be a good inhibitor of herpes virus DNA polymerase (*Helgstrand et al* 1978 *Helgstrand & Öberg* 1978) The structure of PFA is similar to that of phosphonoacetate (PAA) which has the same activity as PFA on herpes virus DNA polymerase (*Helgstrand et al* 1978 *Helgstrand & Öberg* 1978 *Reno et al* 1978) Both structures are similar to pyrophosphate and may interact with a pyrophosphate binding site on the polymerase

Serum specimens for DNA polymerase assay were prepared as described previously (*Nordenfelt & Kjellen* 1975) The serum was taken from three patients

serum (*Nordenfelt & Kjellen* 1975 *Couleru et al* 1973) Electron microscopy studies have been repeated on serum preparations from two of the patients (Ass and EsG) which showed a high number of Dane particles in the pellets with polymerase activity Assay conditions for the polymerase were the same as described earlier (*Kaplan et al* 1973)

the 3 h incorporation was 200–300 counts/min The DNA polymerase activity in the presence of inhibitor is presented as percentage remaining incorporation of [^{3}H]dTTP into acid precipi

TABLE 1 *Inhibition of Dane Particle DNA Polymerase at Different Concentrations of PFA and PAA*

Conc µM	Percentage remaining activity, mean	S D	PFA No of determinations			Percentage remaining activity, mean	S D	PAA No of determinations		
			Patient Ass	Patient EsG	Patient AA			Patient Ass	Patient EsG	Pa[illegible]
10	83 7	22 2	5	3	1	144	60 0	2	1	[illegible]
20	45 6	17 8	7	2	1	–	–	–	–	[illegible]
50	30 1	17 9	5	2	–	153	24 0	2	1	[illegible]
100	11 2	15 3	8	3	1	142	30 0	2	1	[illegible]
200	13 2	–	–	1	–	–	–	–	–	[illegible]
500	5 1	11 2	2	1	1	72 1	17 1	2	1	[illegible]

table radioactivity as compared to the incorporation without inhibitor A control serum from HB_sAg negative persons was always included but did not show any DNA polymerase activity

A summary of the results is given in Table 1 The concentration dependence of the inhibition by PFA is presented and also compared with the effect of PAA The Dane particle DNA polymerase was inhibited to 50% by 20 µM PFA while 20 µM PAA seemed to have a slight stimulatory effect At 100 µM almost all DNA polymerase activity was inhibited by PFA but PAA was still stimulatory A slight inhibition was caused by 500 µM PAA The lack of inhibition of the DNA polymerase by PAA *correlates with earlier results* where no inhibition was seen at concentrations up to 1 mM (*Mao & Robishaw* 1975)

PFA and PAA have similar activities on herpes virus DNA polymerase which is inhibited to 50% at 3 and 7 µM respectively (*Helgstrand et al* 1978 *Helgstrand & Öberg* 1978) A difference in inhibition was observed *with influenza virus RNA polymerase* where PFA was more inhibitory and for vaccinia virus multiplication where PAA was the more active compound (*Helgstrand et al* 1978) Despite the slight difference in structure between PFA and PAA their activities on viral enzymes could evidently be quite different No differences between PFA and PAA in inhibition of cellular DNA *polymerases α β and γ have been observed* (*Helgstrand et al* 1978 *Reno et al* 1978) At concentrations up to [illegible] DNA RNA or [illegible] PFA is necessary to reduce *DNA synthesis* and cell proliferation to 50% in cell cultures and this inhibition is reversible (*Stenberg & Larsson* 1978) A very low toxicity in rats and dogs and no metabolism in mice have been observed for PFA (*Flodh H & Lundstrom J* personal communication 1978) and it does not have the dermal toxicity observed for PAA (*Alenius et al* 1978)

Control of viral hepatitis B is an important medical problem Prevention by passive immunization has met with some success and a vaccine is forseeable in the near future (For review see *Cossart* 1977) A specific chemotherapy against hepatitis B would however be a well needed complement and attempts in that direction have recently been described (*Hirschman & Garfinkel* 1978) The lack of cell toxicity of PFA (*Helgstrand et al* 1978 *Stenberg & Larsson* 1978) and its selective inhibition of Dane particle DNA polymerase suggest that an evaluation of its effect on hepatitis B in animals should be undertaken

We thank Mrs *Eva Miller* for excellent technical assistance The investigation was supported by the *Swedish Medical Council* (project No B77–16X–2865 01)

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cta path microbiol scand Sect B 87 77–83 1979

PRODUCTION AND EVALUATION OF ANTISERA FOR SEROLOGICAL TYPE DETERMINATION OF GROUP-B STREPTOCOCCI BY DOUBLE DIFFUSION IN AGAROSE GEL

NIELS EINAR JENSEN

State Veterinary Serum Laboratory Ringsted Denmark

Jensen N E Production and evaluation of antisera for serological type determination of Group B streptococci by double diffusion in agarose gel Acta path microbiol scand Sect B 87 77–83 1979

A double diffusion technique for serological typing of Group B streptococci is presented The method is val

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me ... are good economy of antisera and easy recognition of weak and/or unspecific precipitates The method is especially useful in epidemiological investigations

Key words Group B streptococci serological type determination antisera *double diffusion*

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Serological typing of Group B streptococci (B str) has a history of more than 40 years On the basis of type specific capsular antigens Lancefield (6 7) distinguished between four serotypes Ia Ib II and III A cross reaction between Ia and Ib strains was caused by a common polysaccharide antigen now des gnated the I

... of Ia and Ibc antigens the so-called Iabc determinant is present in all Type Ic strains (9) The Ibc antigen may be demonstrated also in Type II and Type III strains (2 3 4 15) and in some strains of Serotypes R and X (4) It is only rarely that strains possess only the Ibc antigen (4) Besides the Ibc protein antigen B-str may contain two other protein antigens R and X which are the only antigens by which Type R and Type X can be distinguished Combinations of these antigens with the other antigens mentioned are found with varying frequency (10 11) The antigenic composition of the serotypes of B str as described in the literature is shown in Table I

According to *Jelinkova* (5) the present official system of

... However in epidemiological examinations aimed at elucidating the source and mode of transmission of a B str infection it is essential that the strain in question should be defined as precisely as possible i e both by the type antigen and by possible additional antigens

In the present paper a double diffusion technique is described by which it is possible to run several tests simultaneously and to characterize both B str strains and antisera in terms of the antigens listed in Table I

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A double diffusion technique for serological typing of Group B streptococci is presented The method is valuable both for type determination on a large scale as well as for evaluation of the specificity of type antisera As in previously published methods HCl-extracted antigens are used The preparation of type antisera is described and the reactions of some selected sera are given in figures The advantages of the method are good economy of antisera and easy recognition of weak and/or unspecific precipitates The method is especially useful in epidemiological investigations

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... was caused by a common polysaccharide antigen now designated the Iabc determinant (9) Type Ic (14 15) possesses both the Ia polysaccharide antigen and a protein antigen found in Type Ib strains This protein antigen is now designated Ibc (16) Besides the Ia and Ibc antigens the so-called Iabc determinant is present in all Type Ic strains (9) The Ibc antigen ...

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According to *Jelinkova* (5) the present official system of classification of B str includes strictly speaking only the five polysaccharide types Ia Ib Ic II and III ...

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TABLE 1 *Type Antigen Structure of Group-B streptococci*

Serotype	Polysaccharide antigens	Protein antigens
Ia	Ia, Iabc	(R or X)
Ib	Ib, Iabc	Ibc, (R or X)
Ic	Ia, Iabc	Ibc (R or X)
II	II	(Ibc) (R or X)
III	III	(Ibc) (R or X)
R	–	(Ibc) R
X	–	(Ibc) X
?	–	Ibc, (R or X)

Antigens shown in brackets are not invariably present
? an official serotype designation for »Ibc« strains has not yet been adopted
– polysaccharide antigens are not present
All designations refer to HCl extracted i e denatured antigens

MATERIAL AND METHODS

*B str Reference Strains**)

Type Ia O90
Type Ib H36B
Type Ic A909
Type II 18RS21
Type III D136C
Type R R Compton
Type X X Compton
Group B O90R

The reference strains were subcultured weekly on blood esculin agar plates

Production of Antisera

Vaccine Rabbits and cows were immunized with vaccine prepared from cells grown for 18 hours at 36° C in trypsin digested beef heart broth The cells were killed by treatment for one hour with 2% formalin then washed twice in 0 9% NaCl and resuspended in PBS (pH 7 6) to a concentration four times that of standard 3 of the McFarland s $BaSO_4$ Nephelometer Standards

The sterility of the vaccine was checked by inoculation on to blood agar plates

Goats were immunized with formalin killed vaccines prepared as described by *Lancefield* (1975)

Immunization Schedule

Rabbits Initially five intradermal injections of 0 1 ml cells + 0 1 ml Freund s complete adjuvant were given to the rabbits at 14 day intervals For revaccination two courses of intravenous injection of cells alone were given Each course consisted of five injections given at 2 day intervals and the dosage was as follows 1st injection 1/4 ml 2nd and 3rd injections 1/2 ml 4th and 5th injections 1ml 6th–10th injections 2 ml The interval between the initial vaccinations and the first revaccination and between the 1st and 2nd revaccination was about 3 months

Cows The cows were immunized in accordance with the schedule used for 1st and 2nd revaccination of the rabbits with five times higher doses Revaccination was not carried out

Goats The goats were immunized as described for rabbits by *Lancefield et al* (9)

Storage and preservation of sera Merthiolate (1 10000) was used as preservative The sera were divided into 5 ml portions and stored in frozen state (–20° C) until used While in use (i e for 1 to 6 weeks) they were stored at + 4° C

Absorption of sera Lancefield s method (9) was applied using heat killed cells

Preparation of Extract

The cells were grown in 10 ml Todd Hewitt broth (Difco) for 16–18 hours at 37° C The culture was centrifuged for 10 min at 3000 rev/min The supernatant was decanted and the cells were resuspended in 0 5 ml N/20 HCl Extraction took place at 100° C for 15 min (waterbath) Neutralization was made with Na_2CO_3 (3%) Phenol red was used as indicator The suspension was recentrifuged and the supernatant used as antigen extract

Peptic digestion of extracts Before neutralization 0 1 ml of a 5% pepsin solution (10000 units/g Merck) was added The mixture was incubated at 37° C overnight then neutralized and centrifuged

Preparation and Treatment of Agarose Plates

Details of the preparation of agarose plates the material used and the post treatment and staining have been described by *Weeke* (13)

Briefly the procedure is as follows A 1% agarose solution in diemal buffer (pH 8 6) is poured on to glass plates (100 × 100 × 1 5 mm) in portions of 15 ml The 15 wells (diameter 2 5) are arranged as shown in Fig The centre to centre distance between serum and antigen wells is about 7 mm ($\sqrt{50}$ mm) Rows of eight wells are used for antigen and rows of nine wells for serum Both antigen and serum are applied in amounts of 5 microlitres (Oxford micro syringe) Diffusion is allowed to proceed for 48 hours at 20° C

In one plate it is possible to test seven »unknown« sera against extracts of seven type reference strains and the group reference strain

For type determination of B str strains eight plates are used (Fig 15) each with the same specific antiserum applied to all serum wells Extracts of the strains concerned are placed in identical positions on the eight plates By this arrangement it is possible to type 56 strains at a time using seven known type strains and the group strain for reference

*) The author wishes to acknowledge his indebtedness to Dr *Beate Perch* Statens Seruminstitut Copenhagen for supplying the reference strains

RESULTS

Type Antisera

A total of 38 animals (32 rabbits three goats and three cows) were vaccinated with the type reference strains

Type Ia (Antigens Ia Iabc)

Figures 1 and 2

Five rabbits two goats and one cow were vaccinated with strain 090

The reactions of a serum drawn from a rabbit two weeks after the first series of vaccinations are shown in Fig 1 Antibodies reacting with Type Ia Type Ic and the group antigen (090R) had developed Absorption with X-cells (Fig 2) eliminated the group reaction and made the serum specific for the Type Ia polysaccharide antigen (Types Ia and Ic)

Serum from another rabbit showed no reaction after the initial series of vaccinations with the Ia

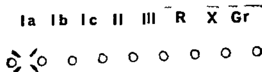


Fig 1 Antiserum Ia unabs

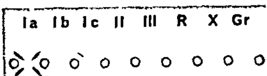


Fig 2 Antiserum Ia abs X

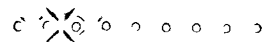


Fig 3 Antiserum Ib unabs

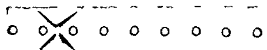


Fig 4 Antiserum Ib abs Ic



Fig 5 Antiserum Ic unabs

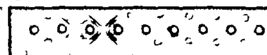


Fig 6 Antiserum Ic abs Ia



Fig 7 Antiserum II unabs



Fig 8 Antiserum II abs X

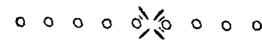


Fig 9 Antiserum III unabs



Fig 10 Antiserum III abs X

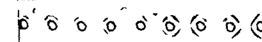


Fig 11 Antiserum R unabs



Fig 12 Antiserum R abs X

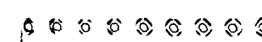


Fig 13 Antiserum X unabs

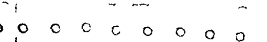


Fig 14 Antiserum X abs Gr

Double diffusion in agarose gel of some selected group B type antisera before (unabs) and after absorption (abs) Arrangements common for all figures

The row of nine wells (middle) contains the serum to be examined and the two rows of eight wells the extracts of the seven type reference strains and the Group B strain The arrangement of the extracts is shown in Fig 1 and 2 only but is identical for all sera tested

reference strain but very weak reactions with extracts of this strain and the group strain after the 1st series of revaccinations Following the 2nd series of revaccinations antibodies for Ia Ib and Ic were demonstrated and the immuno precipitates showed a picture of complete identity The same reactions were obtained after peptic digestion of the extracts thus indicating that the reacting antigen is a polysaccharide Absorption with Ib and Ic cells removes all antibodies from the serum The antibody present in this serum reacts with the Iabc determinant

A third rabbit produced an identical (anti Iabc) serum after the 1st series of revaccinations Sera from the two goats reacted weakly with Type Ia antigen and the group antigen after the 1st series of vaccinations Revaccination of one of them did not enhance the reactions Two rabbits and one cow did not respond to the 1st series of vaccinations and were not revaccinated

Type Ib (Antigens Ib Iabc Ibc)

Figures 3 and 4

Three rabbits and one cow were vaccinated with H36B

Following the 2nd series of revaccinations one rabbit produced antibodies reacting with the Ib polysaccharide antigen the Ibc protein antigen (demonstrated by absorption with Ia cells) the Iabc determinant and the group B antigen (Fig 3) After absorption with Ic cells (Ia Iabc Ibc) this serum reacted specifically with the Ib polysaccharide antigen (Fig 4)

Another rabbit produced an anti Ibc serum after the 1st series of revaccinations Following the 2nd series of revaccinations antibodies against the Iabc determinant were also present but no antibodies against the Ib polysaccharide antigen were detected

The third rabbit produced no antibodies at all

After the 1st series of vaccinations the cow produced antibodies which reacted weakly but specifically against the Ib polysaccharide antigen

Type Ic (Antigens Ia Iabc Ibc)

Figures 5 and 6

Five rabbits and one goat were vaccinated with A909

After the 1st series of vaccinations the goat produced antibodies against the Ibc antigen and following revaccination also antibodies against the Ia antigen The reaction with the Ibc antigen was enhanced after revaccination (Fig 5) This serum was made specific for the Ibc antigen by absorption with Ia cells It has the peculiarity of forming two precipitation lines (Fig 6)

Three of the rabbits produced antibodies reacting weakly against the Ia polysaccharide and strongly against the Ibc protein antigen after the 1st series of vaccinations Two of them were revaccinated twice and following the 2nd revaccination one of the rabbits reacted against the Iabc determinant only while the reactions remained constant for the other Two rabbits produced antibodies against the Iabc determinant after the 1st series of vaccinations

Type II (Antigen II)

Figures 7 and 8

Three rabbits and one cow were vaccinated with 18RS21

Serum from one rabbit reacted weakly but specifically with Type II antigen after the 1st series of revaccinations Following the 2nd series the reaction of the serum became stronger and a reaction with the group antigen also occurred (Fig 7) The group reaction could be removed by absorption with X-cells after which the serum reacted specifically with the reference strain (Fig 8)

Another rabbit produced after the 1st series of revaccinations a serum reacting weakly with II and group B antigens and after the 2nd revaccination also antibodies against X antigens The third rabbit produced antibodies against II R and X antigens after the 2nd revaccination

When the double diffusion technique was used serum from the cow reacted weakly but specifically after the initial series of vaccinations The cow was not revaccinated When tested by the capillary ring precipitation test this serum gave a strong precipitation reaction

Type III (Antigen III)

Figures 9 and 10

Five rabbits were vaccinated with D136C

After the 2nd series of revaccinations two sera reacted with the Type III polysaccharide antigen and with the group antigen (Fig 9) The group reaction could be removed by absorption with X-cells (Fig 10)

In three sera no antibodies could be detected even after the 2nd series of revaccinations

Type R (Antigen R)

Figures 11 and 12

Six rabbits were vaccinated with the R Compton strain Five sera had antibodies against this strain two after primary vaccination and three following revaccination The sera reacted both with the X antigen and with the group antigen (Fig 11) The figure shows that also the group reference strain (090R) contained the R antigen Following absorption with X-cells the sera reacted specifically with the R antigen (Fig 12) No antibodies were detected in serum from the sixth rabbit

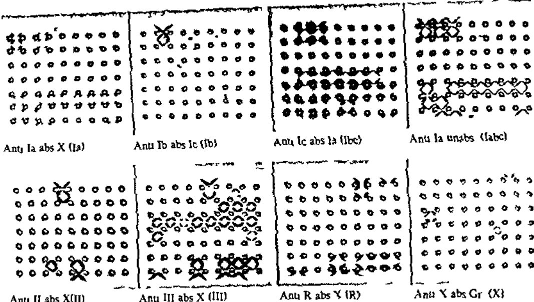


Fig 15 Type determination of 56 Group B strains by double diffusion in agarose gel Extracts are placed in identical positions on the eight plates Rows of nine wells are used for antiserum rows of eight wells are used for the seven type reference strains and the group B strain (upper row) and for the 56 *strains examined*
The antiserum used is indicated for each plate together with the antigen determined in *brackets*

Type X (Antigen X)

Figures 13 and 14

Five rabbits were vaccinated with the X Compton strain

Four rabb...

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present (Fig 13) After removal of these by absorption with R-cells type-specific sera were obtained (Fig 14) One rabbit produced no antibodies at all

Typing of B str

The above mentioned type-specific sera (anti Ia anti Ib anti Ibc anti Iabc, anti II anti III anti R and anti X) were used for typing a number of B str strains of bovine and human origin (see Fig 15) The antigenic composition of the strains examined can be read directly from the stained plates Reading of the ›typing-plates‹ has not presented any difficulties and so far it has been possible to classify 100 % of the human (149 strains) and 98 5 % of the bovine strains (597) examined The results of these investigations will be published in a subsequent paper

Instead of placing type antisera in the wells in every second row an attempt was made to incorporate the antibody in the agarose gel (0 7 ml serum/15 ml gel) With the same arrangement of the wells and extracts of B str in all the »serum« wells 64 different strains can be examined at a time against one serum This method also offers the possibility of quantifying the content of antigen in the strains (radial immuno-diffusion) Preliminary experiments have given promising results

DISCUSSION

No clear-cut picture has been formed with regard to the type of vaccine the method of application the number of injections and the length of the intervals between injections necessary for ensuring the production of a B-str type antiserum suitable for precipitation by double diffusion Individual characteristics of the animals seem to play a major role

Except in the case of Type Ic vaccine most attempts to immunize rabbits by intradermal administration of vaccines containing Freund's complete adjuvant gave disappointing results

Revaccinations were performed by a ...

... for all reference strains

After the initial course of vaccinations two cows inoculated with Type Ib and Type II respectively

produced sera which reacted weakly and slowly *though specifically in double diffusion tests* while they reacted strongly in capillary ring precipitation tests The reverse (*strong reaction in double* diffusion and *weak reaction in capillary ring precipitation tests) was experienced with rabbit sera* The reason for this discrepancy is unknown Since the cows were not *revaccinated it remains an open* question as to whether revaccination could have changed this mode of reaction

Two goats vaccinated with the Type Ia strain *reacted weakly after the* initial course of vaccina tions and revaccination of one of them did not enhance the reaction On the other hand a goat vaccinated and revaccinated with the Type Ic strain (antigens Ia Ibc Iabc) gave a strong reaction with two distinct precipitation lines against the Ibc *protein antigen but only a weak reaction against the* Ia polysaccharide antigen The Ia reaction almost disappeared later *Wilkinson/Eagon* (15) found that the Ibc antigen consisted of two serologically active determinants responsible for the two precipitation lines

The rapid and strong antibody production seen in rabbits vaccinated with Type Ic + Freund s complete adjuvant and in the goat vaccinated with Ic may reflect a better immunological response to this strain than to the other B str type reference strains

In two rabbits *vaccination with Type Ia (090)* resulted in sera reacting with the Iabc determinant *only The same was the case with two Ic sera after* the 1st series of vaccinations and with a Ic and a Ib *serum after the 2nd revaccination* According to *Lancefield et al* (9) antibodies occur less frequently against the Iabc determinant than against the major determinants (Ia and Ib)

However the relatively high concentration of formalin (2% one hour) used in this study to kill the vaccine cells may have influenced the major type specific polysaccharide *Lancefield et al* (9) used a concentration of 0 3% formalin and mention that a higher concentration of formalin (up to 3%) was unsatisfactory for preparing vaccines for specific types of *B str*

The type specific polysaccharides are not present in their *native form in the* HCl extracts (1 8 12 16) In fact the serological typing of B str is based on the demonstration of the denatured antigens occurring in the HCl extracts and provides suffi cient differentiation for epidemiological purposes

The content of HCl-extractable antigens in B str strains is variable as seen from the fact that extracts obtained from different strains by the same treatment yield precipitates of varying density *Quantification of* the antigen content may be possible by the radial immuno-diffusion technique

The variations applied mostly to the Ia polysa charide antigen which is apparently found in ve small quantities in many Ia and Ic strains On th other hand such strains always gave stron precipitation with the Iabc serum thus forming valuable aid in type determination Based on th present knowledge of B str type antigens a strai *lacking the Iabc determinant but reacting with th* Ibc antiserum is sure to be a »pure Ibc« strain an *not a Ic or Ib strain containing a very small amou* of a Ia or Ib polysaccharide antigen

Only *small amounts of serum are required fo* type determination by double-diffusion in agaros gel and turbidity of the serum applied does nc influence the reading of the results Weak precipita tes are observed readily and possible unspecifi precipitates are recognized more easily than in th capillary ring precipitation test *Furthermore* th results need not be read at fixed times and the plates can be stored for later documentation when stained and dried The method is advantageous especially when knowledge of the antigenic composition of greater numbers of B str strains is required e g for epidemiological studies but less so when type determination is needed quickly

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INFLUENCE OF O AND K ANTIGENS ON THE SURFACE PROPERTIES OF *ESCHERICHIA COLI* IN RELATION TO PHAGOCYTOSIS

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Stendahl O Normann B & Edebo L Influence of O and K antigen on the surface properties of *Escher chia coli* in relation to phagocytosis Acta path microbiol scand Sect. B 87 85–91 1979

Strains of *Escherichia coli* with different O and K antigens were investigated with respect to physicochemical surface characteristics and liability to phagocytosis Using two phase partitioning analysis for the surface characterization three main groups of strains emerged Group I (O1 K1 O2 K1 O3 K2ab) showing both smooth hydrophilic O antigens and negatively charged K antigen wh ch rende ed h [illegible]

removed by heat treatment these stra ns became [illegible] ugen when the K antigen was (O28 K– O56 K+ and O118 K–) c [illegible] er strains physicochemical surface effects anc [illegible] that the relation to the properties conveyed by the corresponding O antigens [illegible] luated in

Key words *Escherichia coli* surface properties O antigens K antigens phagocytosis

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The virulence of many enterobacteria in extra intestinal tissue is closely linked to the structure of their O antigen In *Salmonella* (3 18) and *Escherichia coli* (13 17) the complete O antigen with repeating polysaccharide side chain units protects the bacteria from phagocytic uptake and subsequent intracellular killing Mutations S (smooth) → R (rough) that lead to varying defects in the lipopolysaccharide (LPS) structure diminish the resistance to phagocytosis (4 13 18) In fact Rd and Re mutants from *S typhimurium* are phagocytosed to a considerable extent even in the absence of antibody and complement (20)

We have studied previously the surface properties of *S typhimurium* 395 MS (smooth) and certain R mutants derived from it using aqueous two-polymer phase systems and hydrophobic interaction chromatography (10 11 19 20) These experiments revealed a close correlation between general physicochemical surface characteristics such as hydrophobicity and charge and resistance to phagocytosis In these bacteria the LPS seems to convey most of these characteristics

In *E coli* the picture is more complex Although the LPS influences the antigenicity virulence and resistance to phagocytosis (13) the K antigen mostly an acid polysaccharide with varying degrees of polymerisation (9 16) has also been suggested as an impedin (5 6) This antigen constitutes the outermost layer of the bacteria thus giving the surface a strong negative charge which inhibits agglutination with anti-O antibodies (8) complement activation (6) and phagocytosis (6) The

amount of K antigen has also been shown to influence the *in vivo* localization of bacteria in the urinary tract (7) but not in the blood stream (12) However little information is available regarding the relative importance of the O and K antigens The present investigation analyses the surface properties of a series of *E coli* strains with respect to surface characteristics such as charge and hydrophobicity and interaction with phagocytic cells By comparing the surface properties and liability to phagocytosis of living and heattreated bacteria (thus eluting the K antigen) the functional expression of the O and K antigens on the bacterial surface is revealed

MATERIALS AND METHODS

Strains The *Escherichia coli* strains O1 K1 (U5/41) O2 K1 (U9/41) O3 K2ab (U14/41) O4 K3 (U4/41) O6 K2ac (Bi7458/41) O14 K7+ (Su4411/41) O24 K− (E41a) O28 K (K1a) O55 K59 (Su3912/41) O56 K+ (Su3684/41) O111 K58 (Stoke W) and O118 K− (W31) were kindly supplied by Dr *F Orskov* Copenhagen and the O1 K neg strain by Dr *B Kayser* Gothenburg

Cultivation heat treatment and radioactive labelling All strains were kept on agar slants at 4° C before use The bacteria were grown in 10 ml nutrient broth (Difco) at 37° C for 18 h harvested by centrifugation (6000 × g 10 min) and washed in phosphate buffered saline (PBS) pH 7 2 The bacteria were labelled with either ^{51}Cr or ^{125}I as described previously (19) An Autogamma scintillation counter (Intertechnique Plaisir France) was used for the radioactive determination For heat treatment each lot of labelled bacteria was divided into two portions one kept at 4° C and the other in water bath at 70° C for 45 min The bacteria were then washed twice in PBS and suspended in the appropriate buffer

Phase systems and partition analysis The two phase ... 4 4% (w/

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fer pH 7 0 (1) They were prepared from stock solutions of 20% polyethylene glycol 6000 (Carbowax 6000 Union Carbide New York N Y) 20% dextran T500 (Pharmacia Fine Chemicals Uppsala Sweden) 0 1 M tris buffer and distilled water and were allowed to

detailed description see reference (20)

Phagocytosis experiments The phagocytosis procedure *in vitro* has been described in detail previously (18) Briefly polymorphonuclear neutrophil leukocytes (PMNL) were collected from the peritoneal cavity of guinea pigs after injection of 0 2% glycogen The cells were washed twice in Krebs Ringer phosphate buffer with 10 ml glucose (KRG) pH 7 2 suspended in the same buffer and allowed to adhere to cellulose acetate filters (Millipore) on the bottom of plastic petri dishes (1–2 × 10⁷ PMNL/dish Flow Laboratories Irvine Scotland) Non adhering leukocytes and erythrocytes were washed off before the addition of labelled bacteria (5 × 10⁸ bacteria) suspended in KRG with 0 1% bovine serum albumin (Poviet Amsterdam Holland) At indicated intervals filters were removed washed thoroughly in PBS and measured for radioactivity Phagocytosis was expressed as the percentage of added bacteria adhering to the filters The standard deviation in five experiments was ± 8%

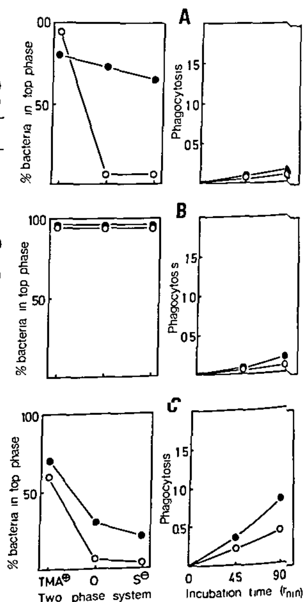


Fig 1 Two phase partitioning (left) and *in vitro* phagocytosis (right) of different *E coli* strains before (○) and after heat treatment (●) (A) *E coli* O1 K1 (B) *E coli* O111 K58 (C) O14 K7 Partitioning is expressed as percentage of added bacteria in the top phase containing 10% TMA PEG only PEG or 10% S PEG Phagocytosis is expressed as percentage of added bacteria associated per filter

TABLE I *Two phase Part t on and Phagocytos s of D fferent* Escherichia coli *Stra ns Treated at 4° and 70°C*

Stra n	Two-phase partition[a] 4° C	70° C	Phagocytosis[b] 4° C	70° C
O1 K1	7	75	0 10	0 20
O2 K1	10	76	0 16	0 38
O3 2ab	20	83	0 12	0 42
O4 K3	60	65	0 26	0 30
O55 K59	68	63	0 29	0 33
O111 K58	98	100	0 10	0 19
O14 K7	12	34	0 40	0 80
O24 K+	10	45	0 39	0 65
O28 K	20	28	0 23	0 30
O56 K+	55	50	0 90	1 07
O118 K	4	3	0 12	0 18

[a] Expressed as percentage of bacteria in the top phase of a two phase system containing 4 4 % PEG and 6 2 % dextran in 0 03 M tris buffer pH 7 0 Mean of at least two experiments run in dupl cate

[b] Expressed as percentage of added bacteria adhering to filters containing 10^6 PMNL after incubation for 90 min Mean of at least two d fferent experiments run in duplicate

RESULTS

Two phase partit oning Using phase systems with negative (S) or positive (TMA+) PEG rich top phase permits analysis of the surface charge expressed on the bacteria. To analyse the contribution of the K antigen to the cell surface characteristics non treated and heat treated bacteria were compared with respect to their surface properties Preliminary experiments revealed that treating the bacteria at 70° C for 45 min gave similar surface characteristics as when bacteria were heated at 100° for 2 h as tested in the two-phase systems Although all the K antigen may not have been eluted the lower temperature was preferred since boiling releases considerable quantities of endotoxin from enterobacteria (3) and other denaturing effects may also occur

Among the tested strains three main groups emerged with respect to the effect of heat treatment on the surface charge The first group (Fig 1A) includes O1 K1 O2 K1 and O3 K2ab all of which

[illegible]

thus indicating strong negative surface charge After heat treatment the bacteria collect in the top phase in an unchar[illegible] 1 are not influenced by either TMA+– or S –PEG thus indicating loss of surface charge In the second group (Fig 1B) which includes the O4 K3 O55 K59 O111 K58 test strains the bacteria collect in the top phase in charged and uncharged systems and heat treatment does not influence the partition pattern (Table I) The third group (Fig 1C) includes O14 K7 and O24 K+ which collect in the bottom phase in uncharged systems and are influenced by TMA+–PEG After heat treatment the bacteria show less affinity for the bottom phase but are still influenced by TMA –PEG thus indicating that they still possess negative surface charge

Strains not fitting into these three main groups are represented by O28 K– O56 K+ and O118 K– Although they and also O14 K7 and O24 K+ belong to chemotype I the LPS of which contains only core type sugars (9) they show strikingly different partition patterns (Fig 2 Table I) O28 K– collect mainly in the bottom phase in the uncharged system and show a strong negative charge independent of heat treatment Viable and heat treated O118 K– bacteria collect in the bottom phase but show no detectable surface charge O56 K+ bacteria collect in the top phase in the uncharged system and more than 90 % in the TMA+–PEG top phase thus indicating negative surface charge However this surface charge is not reduced by heat treatment

These data suggest that the heat-labile properties conferring the negative charge on the *E coli* bacteria are due to different K antigens In order to investigate this further, study was made of the surface properties of some strains with the same O antigen but with varying amounts of K antigen Fig 3 shows the difference in sensitivity to heat treatment between *E coli* O1 KN (K negative) and O1 K1 (K positive) The K positive strain collects in the bottom phase in the uncharged system and is transferred to the top phase by TMA +-PEG, thus showing negative surface charge of the bacteria After heat treatment, the bacteria accumulate in the top phase independent of charged PEG thus showing that the surface charge is lost The K negative bacteria, however, collect in the top phase both before and after heat treatment and are thus devoid of surface charge. Similar patterns were obtained for certain other K positive and K negative strains

In vitro phagocytosis by guinea-pig PMNL All the strains were tested simultaneously with respect to two phase partition and phagocytic uptake Figs 1 and 2 and Table 1 show that the sensitivity of the different strains to phagocytosis correlates with two phase partition both before and after heat treatment The strains in group I (O1 K1 O2 K1 O3 K2ab) are all poorly phagocytosed After heat treatment which results in reduced surface charge they are still poorly phagocytosed though a slight increase is observed for all three strains The heat resistant strains in group II are almost as resistant to phagocytosis after heat treatment as before Strains O14 K7 and O24 K + in group III are more liable to phagocytosis than most of the other bacteria and heat treatment renders the strains even more sensitive to phagocytosis

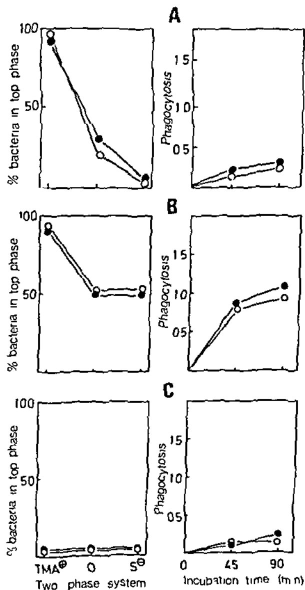


Fig 2 Two phase partitioning (left) and *in vitro* ... ains before (O) and ... O28 K– (B) *E coli* ... o phase partitioning and phagocytosis are expressed as described in legend to Fig 1

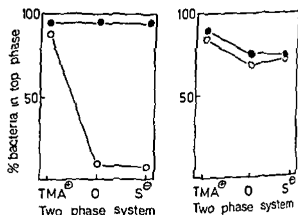


Fig 3 Two phase partitioning of a K positive *E coli* (left) and a K negative *E coli* O1 K1 (right) strain before (O) and after (●) heat treatment Two phase partitioning is expressed as described in legend to Fig 1

The two O1 strains one with K antigen (K1) and one without are poorly phagocytosed both before and after heat treatment

The other strains (O28 K– O56 K + O111 K–) possess differing sensitivity to phagocytosis O28 K– is poorly phagocytosed before and after heat treatment O56 K + is the most phagocytosis sensitive of all the tested strains 0 90% and 1 07% for non treated and heat treated strains respectively Finally, *O118 K– is poorly phagocytosed* whether treated with heat o

DISCUSSION

In *Escherichia coli* both O antigens and K antigens influence the surface properties of the bacteria It is a well known fact that K antigens render the bacteria inagglutinable with anti-O antibody (8) and inhibit phagocytosis (6, 13) The present results indicate a direct anti phagocytic effect of certain O and K antigens Depending on the physicochemical properties of the underlying O antigen the anti phagocytic expression of the K antigen may vary The search for a correlation between the amount of K antigen and different infections may thus be hampered if the O antigen-dependent surface properties of the bacteria are neglected Although the K antigens have not been identified serologically in the present experiments, the loss of negative surface charge after heat treatment has been interpreted as a reduction of K antigen, since this reduction was found in K positive but not in K negative strains (Fig 3)

The partitioning of bacteria in the two-phase systems depends on charge and hydrophobicity (10, 20) Earlier work (16, 17) has shown that phagocytosis-sensitive R mutants of *S typhimurium* collect in the dextran rich bottom phase due to their hydrophobicity and negative surface charge Smooth phagocytosis resistant strains on the other hand collect in the PEG rich top phase due to their hydrophilic and uncharged surface In *S typhimurium* the surface properties are governed primarily by their LPS (20) On the basis of these data, the following interpretation can be put forward concerning the surface properties of the *E coli* strains tested The surface of O1 K1, O2 K1, and O3 K2ab is dominated by the negatively-charged acidic polysaccharide K antigens (16) since the bacteria collect in the dextran rich bottom phase and are attracted to the top phase by TMA$^+$–PEG After heat treatment which elutes the K antigens the negative charge is lost and the bacteria collect in the top phase being inconspicuously influenced by charged polymers thus indicating that they now exhibit uncharged hydrophilic properties presumably S type LPS similar to the smooth *S typhimurium* 395 MS (10) The S (smooth) type of O1 O2 and O3 LPS is further supported by the immunoelectrophoretic patterns (16) and the presence of special sugars such as amino-dideoxyhexoses and rhamnose (9) The surface dominated by the S type LPS (O antigen) retains the resistance to phagocytosis In consequence only a minimal increase in phagocytosis is observed after heat treatment since the presence of the K antigens can add little to the already resistant bacteria Analogously the K negative O1 strain was only slightly more susceptible to phagocytosis than the K positive O1 strain This group of strains which show both a phagocytosisinhibiting smooth LPS and a negatively-charged K antigen has been suggested as the cause of primarily extra intestinal infections such as urinary tract infections and sepsis (14)

The O4 K3, O55 K59 and O111 K58 test strains show partition to the top phase independent of charged polymers and resistance to phagocytosis both before and after heat treatment (Fig 1, Table 1) In these respects they are similar to the non-charged, hydrophilic *S typhimurium* 395 MS (10) The presence of S type LPS in these *E coli* strains is supported further by their content of colitose, rhamnose and amino-dideoxyhexose in the LPS (9) and the immunoelectrophoretic patterns The presence of S type LPS alone may be sufficient to prevent phagocytosis The absence of charge on these test strains, even before heat treatment, agrees with the observation that K58 and K59 are not [illegible]

[illegible] emerged, viz heat treatment caused loss of surface charge, but the bacteria still collected in the bottom phase and retained a certain negative charge Furthermore, the heat treatment enhanced the liability to phagocytosis of these bacteria The negative surface charge retained after heat treatment is similar to *S typhimurium* 395 MR10 (10) an Rd mutant Presence of LPS without S-specific polysaccharide side chain is further supported by the absence of special sugars (chemotype I) (9) and the slight negative charge in the immunoelectrophoretic pattern (16) This non K antigen associated negative surface charge may in fact promote phagocytosis, since a certain minimal negative (or positive?) charge seems to be important in triggering the phagocytic process (*Stendahl* unpublished observation) However, with the negative K antigen linked to the outermost part of the bacterial surface, these bacteria were relatively resistant to phagocytosis [illegible]

[illegible] partition before and after heat treatment) However these two strains still show striking differences in liability to phagocytosis The reason for this is unknown althouth quantitative differen-

ces in surface charge and hydrophobicity due to acidic saccharides in the O antigen (14) may be of importance In fact, preliminary experiments show that O56 K+ is more liable to hydrophobic interaction than the other strains The partition of O118 to the bottom phase is not influenced by heat treatment or charged polymers This strain thus has a hydrophobic uncharged surface and exposes no K antigen In spite of the hydrophobic surface characteristics it is poorly phagocytosed

To evaluate the effect of the K antigens on phagocytosis the properties of the co-existent O antigen must by taken into account In bacteria with an S type O antigen the K antigen may add little to the phagocytosis resistance On the other hand when K antigen is present on bacteria with R-type O antigens it impedes the attachment to the mammalian cell membranes Although the K-antigen did not contribute significantly to the phagocytosis resistance of O1 K– O2 K1 and O3 2ab in our serum-free *in vitro* system, it may have relevance *in vivo* as impedin by inhibiting antibody binding and complement activation (5 6) However *Björksten et al* (2) reported recently lack of correlation between the K1 antigen and serum interaction

In general, the present two phase analysis correlated well with the immunoelectrophoretic analysis of extracted O and K antigens (14–16) We conclude that the negative surface charge due to K antigens is a virulence attribute which enhances the resistance to phagocytosis However S type surface LPS also prevents phagocytosis Thus relevant correlation between virulence and the K antigens can not be drawn without taking into consideration the surface properties conveyed by the underlying O antigens

The technical assistance of *Kerstin Hagersten* is gratefully acknowledged This work was supported by grant 16X–2183 from the *Swedish Medical Research Council*

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Acta path microbiol scand Sect B 87 93–96 1979

SURFACE CHARACTERISTICS OF *ESCHERICHIA COLI* STRAINS IN RELATION TO DEVELOPMENT OF BACTERAEMIA

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Stendahl O & Normann B Surface characteristics of *Escherichia coli* strains in relation to development of bacteraemia Acta path microbiol scand Sect B 87 93–96 1979

Three groups of *E coli* bacteria isolated from blood urine and faeces were analysed with respect to physicochemical surface properties in aqueous polymer two-phase systems Eighty-eight per cent of the bacteria isolated from blood cultures showed affinity for the dextran rich bottom phase whereas 60% and 30% of the bacteria isolated from urine and faeces respectively collected to the same phase Further two phase analysis indicated that the bacteraemia strains exposed more negative surface than the other groups of bacteria and that this charge was reduced after heat treatment (70° C 40 min) These results thus indicate that the strains causing bacteraemia possess similar surface properties and may have been selected from the more heterogeneous group of bacteria found in urine and faeces It is further conceivable to propose that bacteria found in the blood expose more heatsensitive negatively charged K antigen than the other groups of bacteria

Key words *Escherichia coli* strains surface characteristics bacteraemia

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Earlier studies of the surface antigens of *Escherichia coli* indicated that they exerted antiphagocytic activity linked to quantitative and qualitative differences in the somatic O antigen and capsular K antigens (4 9 11) Based on immunoelectrophoretic analysis of the antigens Ørskov et al (15 16) could separate the *E coli* strains into certain groups which were related to the pathogenicity of the different strains They thus proposed that bacteria which may invade the tissue and cause extra intestinal disease had negatively-charged surface antigens Quantitative analysis of the K antigens indicated that K rich strains were more likely to produce renal involvement than [illegible]
eve [illegible]
for [illegible]

str [illegible] coli containing large quantities of K antigen in bacteraemia (8) may be explained partly by the fact that the virulence appears to depend more on the overall physicochemical surface properties of the bacteria than on the quality of the individual O or K antigens (14) We have adapted a technique that permits analysis of the overall surface properties of *E coli* strains in relation to resistance to host defense mechanisms (14) By studying the partition patterns of bacteria in different aqueous polymer two phase systems it is possible to reveal the surface properties of the bacteria such as charge and hydrophobicity/hydrophilicity properties which are of great importance with respect to virulence in other enterobacteria such as *Salmonella typhimurium* (7 13) Earlier studies have shown that the K antigen expressed negative surface charge on the bacteria but its relevance as a virulence attribute depends also on the underlying O antigens (14) The [illegible]

of the two groups of bacteria (Fig 1B) These results indicate that the strains causing septicaemia possess similar surface properties and may have been selected from the heterogeneous group of bacteria residing in urine or in the intestine

The properties governing the partitioning of bacteria to the dextran rich bottom phase are surface charge and hydrophobicity (7 13) To evaluate differences in surface charge the partition patterns were studied using two-phase systems with positively (TMA +–PEG) charged top phase In Fig 2 the negative surface charge on the bacteria is expressed as the difference in affinity for the positively-charged and the uncharged top chase These data (fig 2A) show that bacteria isolated from blood express more negative surface charge than bacteria isolated from urine and faeces Further more the negative surface charge was sensitive to heat treatment as shown in Fig 2B since the difference in affinity for the charged and uncharged top phase was decreased after heat treatment Heat treatment did not alter the surface charge of the other groups of bacteria to the same extent although certain individual strains lost some surface charge

DISCUSSION

The present study supports the view that the *E coli* strains which cause extraintestinal infections such as bacteraemia have similar physicochemical sur face properties with respect to surface charge and may constitute a selective group of bacteria nor mally residing in the intestine The majority (88%) of the strains isolated from the blood as compared to 60 and 30% of the strains isolated from urine and faeces respectively collected to the dextran rich bottom phase The affinity for the phase was due primarily to increased surface charge since the strains from blood exposed more negative surface charge (Fig 2A) than the other strains and since the difference in surface charge and affinity for the dextran rich bottom phase was reduced after heat treatment (Fig 1B) It would thus appear that a property selecting bacteriaemic strains might de pend on heat sensitive and negatively-charged surface structures In previous studies (14) these properties have been identified as the capsular acidic K antigens It is thus conceivable to propose that the bacteria found in the blood expose more K antigens than the strains isolated from urine or faeces However in addition to the antiphagocytic proper ties of the K antigen other surface properties such as fimbria and glycoproteins may mediate specific recognition between bacteria and mammalian cells

It has been found previously that *E coli* strains causing renal involvement contained more K antigen than those causing urinary bladder infec tions (3 5) Furthermore certain K antigens (K1 K2 K3 K12 and K13) were particularly common in strains causing pyelonephritis in children (6) In septic neonatal meningitis the K antigen was most common (10) However no such correlation was found between type or amount of K antigen of *E coli* strains causing bacteraemia (8) These earlier investigations have studied the amount of K antigen eluted from the different strains but have not considered the relative importance of the K antigen to influence the physicochemical surface properties of the bacteria in relation to other surface proper ties Depending on the properties of the underlying structures the significance of the K antigen may vary even though the same amount of antigens are present on the bacteria Considering the properties of the O antigens there appears to be no dominance of smooth strains since some of the septic strains show partitioning patterns similar to strains like O14 (14)

In this study we did not relate the urinary strains to the clinical picture of the patient Thus these strains may be heterogeneous since bacteria from different types of urinary tract infection (e g acute pyelonephritis chronic pyelonephritis asymptoma tic bacteriuria) have shown relationship to different K antigens (6) Neither have the K and O antigens of the strains been analysed The physico-chemical differences between the urinary strains may there fore either be due to quantitative or qualitative differences of the surface antigens Nevertheless heat-sensitive negative surface properties seem to be virulence attributes in the development of bacterae mia Identification of these surface structures their reaction with humoral factors such as the comple ment system (2) and the effect on the interaction with phagocytic cells and also their relationship to different types of urinary tract infections still remain to be elucidated

The technical ass stance of *Kerst n Hagersten* is grate fully acknowledged This work was supported by grant 16X 2183 from the *Swed sh Med cal Research Counc l*

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expression of K antigen on the bacterial surface to the overall physicochemical properties of the bacteria without considering the amount of K antigens *per se*

MATERIALS AND METHODS

Bacterial strains Strains of *Escherichia coli* collected at random were used for the present study They were isolated at the Clinical Bacteriological Department University Hospital University of Linkoping Linkoping from blood (25 strains) urine (15 strains) and faeces (10 strains) The bacterial strains isolated from blood were considered to be of clinical importance and were never regarded as contaminants The urinary and faecal strains were chosen at random and data concerning the patients were not recorded Only one culture per person was included in the study All isolates were identified as strains of *E coli* on the basis of colony morphology and routine biochemical tests All strains were kept at 4° C on deep agar slants cultured on blood agar plates and checked for contamination before being tested

Cultivation and radioactive labelling The bacteria were transferred from blood agar plates to 10 ml of nutrient broth (Difco) incubated overnight at 37° C harvested by centrifugation washed three times in phosphate buffered saline solution (PBS) and labelled with $Na^{125}I$ as described previously (12) After labelling each strain was divided into two portions one was kept at 4° C and the other was placed in a water bath at 70° C for 40 min The bacteria were then washed twice in PBS and diluted to 10^9 bacteria per ml in PBS

Two phase analysis Two phase systems were prepared from stock solutions of 20 % (w/w) polyethylene glycol (PEG) 6000 (Carbowax 6000 Union Carbide New York) 20 % (W/w) dextran T500 (Pharmacia Fine Chemicals Uppsala Sweden) 0 1 M Tris buffer pH 7 0 and distilled water (1) The final phase systems contained 4 4 % PEG and 6 2 % dextran in 0 03 M Tris buffer To analyse negative surface charges 10 % of the PEG was replaced by bis trimethylamino (TMA)+–PEG (13) After equilibration at 4° C overnight 2 ml of the PEG rich top phase and 2 ml of the dextran rich bottom phase were pipetted into test tubes To each tube was then added 0 1 ml of labelled bacteria The tubes were inverted repeatedly for mixing and the phases were allowed to settle for 20 min at 4° C From each phase 0 5 ml samples were carefully withdrawn to measure the percentage of added radioactivity that collected to each phase The radioactivity was measured in an auto gamma scintillation counter (Intertechnique Plaisir France)

RESULTS

The three groups of bacteria were analysed in two phase partition before and after heat treatment Fig 1A shows the partitioning patterns of the bacteria in an uncharged system containing dextran and PEG

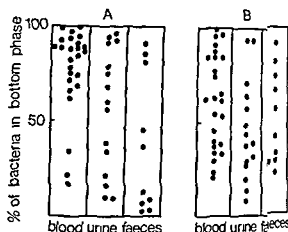


Fig 1 Two phase partitioning of ^{125}I labelled *E coli* strains isolated from blood urine and faeces expressed as percentage of added bacteria collecting to the dextran rich bottom phase before (A) and after (B) heat treatment at 70° C for 40 min Phase composition 6 2 % dextran 4 4 % PEG and 0 03M Tris buffer pH 7 0

The majority 22 out of 25 (88 %) of the bacterial strains isolated from the blood collected primarily to the dextran rich bottom phase whereas strains isolated from urine and faeces collected more at random either to the dextran rich bottom phase or to the PEG rich top phase However after heat treatment the number of strains from blood collected more at random between the top and bottom phase and were similar to the partitioning

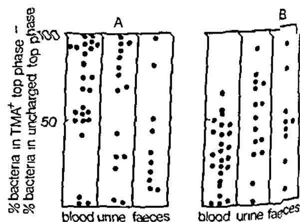


Fig 2 Two phase partitioning of ^{125}I labelled *E coli* strains isolated from blood urine and faeces The partition is expressed as the difference in percentage of bacteria collecting to the TMA PEG rich and to the uncharged PEG rich top phase before (A) and after (B) heat treatment [illegible] ons 6 2 % dextran [illegible] or 6 2 % dex[illegible] substituted with TMA + PEG in 0 03M Tris buffer pH 7 0

Acta path microbiol scand Sect B 87 97–101 1979

CULTIVABLE MYCOBACTERIA IN SPHAGNUM VEGETATION OF MOORS IN SOUTH SWEDEN AND COASTAL NORWAY

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Kazda J Muller K & Irgens L Cultivable mycobacteria in sphagnum vegetation of moors in south Sweden and coastal Norway Acta path microbiol scand Sect B 87 97–101 1979

Intact sphagnum vegetation from moors in south Sweden and coastal areas of west Norway contained cultivable mycobacteria in 32% and 30% of the specimens respectively This frequency of specimens is lower than the 50% previously found in the partly altered moors of northwestern Germany but the Scandinavian moors contained a larger variety of species On both intact and altered moors *M chelonei* and *M sphagni sp nov* were found the latter a homologous group of 151 strains In south Sweden the highest frequency was found in *S balticum S recurvum S tenellum* and *S compactum & molle* (40–65%) In coastal Norway the highest frequency was found in *S rubellum* (48%) which offers favourable conditions for the accumulation of solar energy due to the red brown colour in the upper parts Combined with a high humidity in coastal Norway in summer this may contribute to the growth of mesophilic mycobacteria A significant affinity of *M chelonei* to *S tenellum* was stated

Key words Mycobacteria sphagnum vegetation leprosy

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It has been ascertained that sphagnum vegetation of moor biotopes provides a suitable environment for the reproduction of mycobacteria (*Kazda* 1977 1978 b) Accumulation of solar heat directly under the surface of the vegetation generates a temperature up to 28°C higher than the air temperature offering favourable conditions for the growth of mesophilic microorganisms over an extended period of time (*Firbas* 1931 *Jensen* 1961 *Rudolph* 1964 *Muller* 1965) The sphagnum plants have no roots and no internal capillary system (*Mägdefrau* 1935 *Overbeck* 1975) and circulation of useful nutrients occurs at the surface where the mycobacteria are living in the so called »grey stratum« (*Burgeff* 1961) Important nutrients are carbohydrates above all fructose and amino acids especially glumatic and aspartic acid (*Morton & Broadbent* 1955 *Waksman* 1952 *Black et al* 1955 *Theander* 1954) The ion exchange system localized in the sphagnum cell wall is a type of a natural ion exchange material with a continuous circulation of substances arising from the peaty degradation of the plants (*Brehm* 1971 1975) This system provides fast acidification of the environment (*Anschutz & Gessner* 1954 *Puustjarvi* 1959) Previous investigations have been conducted in moors partly altered by cultivation (*Kazda* 1977) This work concentrates on the isolation of mycobacteria from sphagnum vegetation of intact moors in south Sweden and coastal areas in western Norway

MATERIAL AND METHODS

Moor Biotopes

The *Komosse* sphagnum bog covering an area of 45 km² about 30 km southwest of Jonkoping (57°43 N lat, 13°20 E long) belongs to the largest intact bog complex in south Sweden It is divided into several parts by numerous rills pools and moraines (*Oswald* 1923

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TABLE 2 *Isolation of Cultivable Mycobacteria in Various Species of Sphagnum Vegetation in Coastal Norway*

Sphagnum species	Specimens examined[a]			Strains isolated	Average concentration of mycobacteria in positive specimens
	Total	Positive specimens			
	Number	Number	Per cent	Number	Counts per gram pressed vegetation
S papillosum	45	17	37 8	25	4 019
S rubellum	29	14	48 3	19	1 088
S cusp datum	10	2	20 0	2	182
S compactum	9	2	22 2	2	377
S imbricatum	3	2	66 7	3	310
S plumulosum	1	1	100 0	1	388

[a] In addition 8 negative specimens of *S tenellum S apiculatum S fuscum S palustre* and *S magellanicum*

previously stated the chromatographic comparison of mycolic acid indicates the association to the genus *Mycobacterium* (*Minnikin et al* 1975) The common moor mycobacterium was designated *M sphagni sp nov* and the mycobacterium found only in Scandinavia was designated *M komosseum sp nov* according to a new classification (*Ka da to be published*)

Chi square test was applied in comparing frequencies

RESULTS

Of the specimens of *S balticum S recurvum* and *S tenellum & S molle* found in the Komosse moor 40–56% contained cultivable mycobacteria For species localized on the relatively dry hillocks such as *S rubellum S magellanicum* or *S imbricatum* a frequency of positive specimens of only 15–20% was found (Table 1) In the coastal region of Norway on the other hand the highest frequency of positive specimens was found in *S rubellum* – positive in 48% of the samples followed by *S papillosum* with 38% (Table 2) The difference between frequency of positive specimens in *S rubellum* collected in Sweden and Norway was significant ($p < 0\ 05$)

Compared with the German moors which have been partly altered by agriculture the Scandinavian

TABLE 3 *Isolation and Differentiation of Cultivable Mycobacteria on Scandinavian and German Moors*

Sphagnum bogs	Specimens examined		Strains isolated					
	Total	Positive specimens	Total	*M sphag sp nov*	*M chelonei*	*M gordonae*	*M komosseum*	Others[c] and not differ
	Number	Number Per cent	Number Per cent	Number Per cent	Number Per cent	Number Per cent	Number Per cent	Number Per cent
Komosse (Sweden)	310[a]	99 31 9	143 100 0	67 46 8	10 7 0	16 11 2	14 9 8	36 25 2
Coastal region of Norway	132[b]	39 29 6	51 100 0	12 23 5	4 7 8	12 23 5	4 7 8	19 37 3
Sphagnum bogs in northwest Germany (*Ka da* 1977)	147	73 49 7	110 100 0	72 65 5	25 22 7	0 0	0 0	13 11 8

[a] Included 90 specimens of mosses other than sphagnum
[b] Included 27 specimens of Rhacomitrium lanuginosum
[c] Others *M flavescens M vaccae M diernhoferi*

Overbeck 1975) An area 2 km northwest of Morko between Lake Bjornsjon and Marlebacken was chosen as the site for the investigation Specimens of hydrophilic sphagnum species were collected in the Slattmossen moor from a complex containing numerous rills and pools

In the coastal region of Norway selection of sphagnum biotopes was based on information on former incidence of leprosy (*Irgens & Bjerkedal* 1973)

These biotopes included
1) Soligenous moor on the island of Sotra above Fjeldberg (60°15 N lat 5°3 E long)
2) Soligenous moor above Skadal (61°19 N lat 5°26 E long)
3) Ombro soligenous moor above Skadal (61°19 N lat 5°26 E long)
4) Soligenous moor near Gjerde (61°25 N lat 5°15 E long)
5) Soligenous moor above Stafnes (61°23 N lat 5°10 E long)
6) Soligenous moor on the island of Hitra (near Trondheim) (63°35 N lat 8°39 E long)
7) Soligenous moor on the Flora moor complex near Risvollen (63°32 N lat 11°40 E long)

Selection of Sphagnum Specimens

From the biotopes in Sweden 20 specimens of each of the following species were taken *S imbricatum S magellanicum S rubellum S papillosum S fuscum S cuspidatum S tenellum S balticum S recurvum S acutifolium* and *S compactum & S molle*

In Norway the selection of sphagnum specimens was based on the occurrence of the species in the region concerned A total of 45 specimens of *S papillosum* 29 of *S rubellum* 10 of *S cuspidatum* 9 of *S compactum* 3 of each of *S imbricatum* and *S fuscum* 2 from *S palustre* and 1 each of *S plumulosum S tenellum S apiculatum* and *S magellanicum* was collected

The specimens were handled with sterile plastic gloves transported to the field laboratory in sterile plastic bags and refrigerated (0–+4°C) until they could be examined In the laboratory a specimen of the vegetation was placed in a 20 ml plastic syringe and moistened with a 50 50 mixture of heat sterilized water and distilled water After 10 minutes the water was pressed out of the samples which were weighed The fluid was centrifuged and the sediment resuspended in 2 ml of 0 9% NaCl Petri dishes containing Middlebrook 7H10 agar medium with 4% beef serum were inoculated with a series of diluted suspensions (10 1 to 10^{-4}) These were incubated for 5 weeks at 31°C The colonies were differentiated according to their morphology Smears were stained by the Ziehl Neelsen method Colonies of acidfast bacteria were counted isolated and grown in subcultures Strains demonstrating true branching or an irregular weak acid fastness were not included in the further differentiation of mycobacteria

The examination of the biological and biochemical properties of the isolated strains followed with some modifications the procedure set forth in Bergeys Manual (1974) The following properties were evaluated growth rate at 22 31 and 37°C nitrate reductase (*Bonicke* 1962) Tween hydrolysis (*Wayne* 1966) amidases (*Bonicke* 1962) and the utilization of glucose arabinose dulcitol fructose galactose mannitol mannose sorbitol and xylose through carbo hydrate nitrite reductase (*Bonicke & Kada* 1970)

The mycobacterial strains were classified according to a taxonomic system introduced by *Saito et al* (1977) As

TABLE I *Isolation of Cultivable Mycobacteria in Various Species of Sphagnum Vegetation on Komosse moor*

Sphagnum species	Positive specimens[a]		Strains isolated	Average concentration of mycobacteria in positive specimens
	Number	Per cent	Number	Counts per gram pressed vegetation
S balticum	13	65 0	21	77
S recurvum	10	50 0	18	2 594
S tenellum	9	45 0	12	105
S compactum & S molle	8	40 0	11	318
S papillosum	7	35 0	7	921
S cuspidatum	6	30 0	7	28
S fuscum	6	30 0	4	1 051
S acutifolium	5	25 0	8	222
S imbricatum	4	20 0	8	574
S magellanicum	4	20 0	6	166
S rubellum	3	15 0	1	21

[a] Total number of specimens per sphagnum species 20

TABLE 2 *Isolation of Cultivable Mycobacteria in Various Species of Sphagnum Vegetation in Coastal Norway*

Sphagnum species	Specimens examined[a]			Strains isolated	Average concentration of mycobacteria in positive specimens
	Total	Positive specimens			
	Number	Number	Per cent	Number	Counts per gram pressed vegetation
S papillosum	45	17	37 8	25	4 019
S rubellum	29	14	48 3	19	1 088
S cuspidatum	10	2	20 0	2	182
S compactum	9	2	22 2	2	377
S imbricatum	3	2	66 7	3	310
S plumulosum	1	1	100 0	1	388

[a] In addition 8 negative specimens of *S tenellum S apiculatum S fuscum S palustre* and *S magellanicum*

moor mycobacterium was designated *M sphagni sp nov* and the mycobacterium found only in Scandinavia was designated *M komosseum sp nov* according to a new classification *(Kazda to be published)*

Chi-square test was applied in comparing frequencies

RESULTS

Of the specimens of *S balticum S recurvum* and *S tenellum & S molle* found in the Komosse moor 40–56% contained cultivable mycobacteria For species localized on the relatively dry hillocks such as *S rubellum S magellanicum* or *S imbricatum* a frequency of positive specimens of only 15–20% was found (Table 1) In the coastal region of Norway on the other hand the highest frequency of positive specimens was found in *S rubellum* – positive in 48% of the samples followed by *S papillosum* with 38% (Table 2) The difference between frequency of positive specimens in *S rubellum* collected in Sweden and Norway was significant ($p < 0.05$)

Compared with the German moors which have been partly altered by agriculture the Scandinavian

TABLE 3 *Isolation and Differentiation of Cultivable Mycobacteria on Scandinavian and German Moors*

Sphagnum bogs	Specimens examined		Strains isolated					
	Total	Positive specimens	Total	*M sphag sp nov*	*M chelonei*	*M gordonae*	*M komosseum*	Others[c] and not differ
	Number	Number Per cent	Number Per cent	Number Per cent	Number Per cent	Number Per cent	Number Per cent	Number Per cent
Komosse (Sweden)	310[a]	99 31 9	143 100 0	67 46 8	10 7 0	16 11 2	14 9 8	36 25 2
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Sphagnum bogs in northwest Germany (*Kazda 1977*)	147	73 49 7	110 100 0	72 65 5	25 22 7	0 0	0 0	13 11 8

[a] Included 90 specimens of mosses other than sphagnum
[b] Included 23 specimens of Rhacomitrium lanuginosum
[c] Others *M flavescens M vaccae M diernhoferi*

TABLE 4 *Distribution of Cultivable Mycobacterial Species within Various Species of Sphagnum Vegetation on Komosse Moor*

Sphagnum species	Strains isolated					
	Total	*M sphagni sp nov*	*M gordonae*	*M chelonei*	*M komosseum sp nov*	Not differentiated
	Number Per cent	Number Per cent	Number Per cent	Number Per cent	Number Per cent	Number Per cent
S balticum	21	15	3	0	2	1
	100 0	71 4	14 3	0	9 5	4 8
S recurvum	18	10	2	0	2	4
	100 0	55 6	11 1	0	11 1	22 2
S tenellum	12	1	0	5	2	4
	100 0	8 3	0	41 7	16 7	33 3
S compactum & S molle	11	5	1	0	3	2
	100 0	45 5	9 1	0	27 3	18 1
S papillosum	7	3	1	0	0	3
	100 0	42 9	14 2	0	0	42 9
S cuspidatum	7	4	0	0	1	2
	100 0	57 1	0	0	14 3	28 6
S fuscum	4	1	1	1	0	1
	100 0	25 0	25 0	25 0	0	25 0
S acutifolium	8	3	0	2	0	3
	100 0	37 5	0	25 0	0	37 5
S imbricatum	8	1	1	1	0	5
	100 0	12 5	12 5	12 5	0	62 5
S magellanicum	6	5	0	0	0	1
	100 0	83 3	0	0	0	16 7
S rubellum	1	0	0	0	0	1
	100 0	0	0	0	0	100 0

moors showed a lower frequency of positive specimens, 50% for Germany, 30% for Sweden and 32% for Norway (Table 3) However, several species such as *M gordonae M vaccae M diernhoferi* and a hitherto unidentified group of mycobacteria labelled as *M komosseum sp nov* occurred only in the Scandinavian sphagnum vegetation

In almost all types of sphagnum the species most frequently isolated was *M sphagni sp nov* (Table 4), a homologous group of 151 strains An exception was *S tenellum* where only 8 3% of the strains represented *M sphagni* while 41 7% represented *M chelonei*

The frequency of *M chelonei* was significantly higher in *S tenellum* than in other sphagnum types, ($p < 0.05$) *M chelonei* was isolated also in *S fuscum S acutifolium* and *S imbricatum* but was absent in the remaining types of sphagnum *M komosseum sp nov* which occurred most frequently in *S compactum & S molle* (27,3%), was isolated also in *S balticum S recurvum, S tenellum* and *S cuspidatum* This species seemed to prefer hydrophilic sphagnum types *M gordonae* occurred in several types of sphagnum, without evident preference for any type

DISCUSSION

The importance of sphagnum vegetation as a source of mycobacteria is evident This is clarified by finding cultivable mycobacteria in sphagnum specimens, and by the demonstration of growth of a lot of mycobacterial species in the grey stratum of sphagnum vegetation after experimental inoculation (*Kazda* 1978 b) The difference in frequency of positive specimens of *S rubellum* collected in south Sweden (15%) and coastal Norway (48%), indicates the influence of climatic factors especially humidity According to the »Atlas de climate de Norwege« (*Mohn* 1921) the maximal relative humidity on the Atlantic coast of Norway has been recorded during the summer In other parts of

Norway and south Sweden the highest humidity occurs in winter *S rubellum* offers favourable conditions for accumulation of solar energy due to the red brown colour in the upper part of the plant *M sphagni sp nov* may be considered as permanent in the sphagnum vegetation while *M komosseum sp nov* has as yet been isolated only in intact Scandinavian moors It may be of interest to clarify whether this species occurs only on the Scandina vian peninsula or also in intact moors on the European continent The finding of an affinity of *M chelonei* to *S tenellum* should be further examined

It is interesting to note that most suitable conditions for mycobacterial growth in sphagnum vegetation were found in localities in Norway with formerly high leprosy incidence rates A study on the occurrence of non cultivable mycobacteria in this vegetation is in progress

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THE INTERACTION OF PENICILLIN AND CHLORAMPHENICOL AGAINST MENINGOCOCCI *IN VITRO*

OTTO SOLBERG[1] and BJÖRG MARIT ANDERSEN[2]

Solberg O & Andersen B M The interaction of penicillin and chloramphenicol against meningococci *in vitro* Acta path microbiol scand Sect B 87 103–107 1979

Combinations of penicillin and chloramphenicol are frequently used initially in the treatment of bacterial meningitis The simultaneous effects against meningococci of these two drugs were examined *in vitro* in a chemically defined proteinfree medium The investigation was performed with different combinations of the antibiotics including optimal concentrations In most instances penicillin and chloramphenicol seemed to have an additive but not an iso additive effect No antagonism was found and only one out of nine strains showed indifference

Key words *Neisseria meningitidis* combinations of antibiotics isobologram

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Received 13 vi 78 Accepted 19 x 78

Combinations of antibacterial drugs may for instance be used as emergency treatment of serious infections before bacteriological examinations are completed Combinations of two or three ...

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the action of the bactericidal drug requires bacterial growth (8 10 12) *In vitro* and *in vivo* studies have indicated that penicillins and tetracyclines act antagonistically (7 10) *In vitro* chloramphenicol may interfere with the effect of benzylpenicillin against streptococci (8 9 16)

The purpose of this study was to examine the simultaneous effects of penicillin and chloramphenicol against meningococci in a chemically defined protein free medium where only minimal amounts of other interfering factors are present

MATERIALS AND METHODS

Bacterial strains Nine isolates were studied eight from patients with meningococcal infections and one from a healthy carrier (strain 247) Five were serogroup B (270 840 3967 8884 and 9181) two serogroup A (714 and 1821) one serogroup C (837) and one serogroup Y (247) All were resistant to sulphonamide and sensitive to benzylpenicillin ampicillin erythromycin and chloramphenicol (AB Biodisc on heated blood agar in 5 % CO_2) The isolates were typical meningococci in terms of morphology growth and acid production from sugars The strains were received from the Department of Microbiology Ulleval Hospital Serogrouping of the strains was kindly verified by Dr *E Holten* Oslo Since isolation the ...

blood agar

Medium

(1 13) w

bicarbonat- ... glucose were dissolved in distilled water to 400 ml The pH was adjusted to 5 8 with 1 M

NaOH before sterile filtration by Gelman cellulose triacetate membrane filter of pore size 0 2 μm

The following solutions were prepared and added successively to solution a

b) 0 2 mg cocarboxylase 0 1 g L glutamine and 0 1 mg vitamin B_{12} in 50 ml distilled water filtered sterilely

c) 1 0 mg haemin (X factor) dissolved in 1 25 ml 1 M NaOH and 2 5 mg NAD (nicotinamide adenine dinucleotide V factor) diluted in distilled water to 50 ml filtered sterilely

d) 0 02 g *iron chloride* $FeCl_3 \cdot 6H_2O$ *in 50* ml distilled water filtered sterilely

e) 10 0 g agar Oxoid L 11 and 3 g soluble starch (Difco) in distilled water to 400 ml autoclaved at 120° C *for* 15 minutes

f) 1 5 g disodium hydrogen phosphate $Na_2HPO_4 \cdot 12H_2O$ dissolved in 50 ml distilled water autoclaved at 120° C for 15 minutes

The final pH of this medium was 7 4 ± 0 1

Checkerboard titration of antibiotics Benzylpenicillin Na (A/S Apothekernes Laboratorium Oslo lot 143) and chloramphenicol (Norsk Medisinaldepot Oslo lot 711201) were diluted in sterile distilled water 2 5 ml of *each dilution was added to 45 ml agar medium* and finally poured into a Petri dish (90 × 15 mm) The antibiotics were filtered sterilely by membrane filter 0 2 μm before being added to the agar medium

These dilutions yielded test combinations from 0 010 to 0 042 μg/ml benzylpenicillin and from 0 05 to 0 70 μg/ml chloramphenicol

The inoculates were prepared from growth on heated blood agar (cultivated in an atmosphere containing 5% CO_2 for 18 hours) with a platinum loop into sterile 0 9% *NaCl mixed with a Rotamixer and adjusted to an optical* density (O D) of 0 600 at 620 nm measured in a Duran tube (16 × 160 mm) in Hitachi spectrophotometer model 101 This suspension was equivalent to about 10^9 colony forming units/ml (CFU/ml) For each strain two dilutions of approximately 5 × 10^4 and 10^6 CFU were transferred with a Denley multipoint inoculator to two parallel agar plates The agar plates were read after incubation in 5% CO at 37° C for 18–24 hours The minimal inhibitory concentration (MIC) was defined as *the lowest concentration giving at least 99% growth* reduction

Definitions of combined antibacterial effect The most common combined antibacterial effect is *indifference* where the activity of the combination is equal to that of the more active member of the pair with no contribution from the second less active drug (7) *Synergism* denotes a significantly (four times or more) greater effect of the drug combination than the sum of both drugs An *additive effect* occurs when the combined action is greater but not significantly greater than the sum of both drugs when used alone *Iso addition* occurs when the combined action is equivalent to the sum of the actions of each drug when used alone (11) *Antagonism* occurs when the combined action is significantly (four times) less effective than that of the more active agent when used alone (7)

Isobologram To compare two drugs with different activity against a bacterial strain the MIC of each of the drugs in the various ratios may be expressed as a decimal fraction of the MIC of the respective drug when acting alone This decimal fraction is *termed »fractional* inhibitory concentration« (FIC) The results can be presented graphically as an »isobologram (2)

RESULTS

The MICs of the nine strains studied were 0 018-0 030 μg/ml for benzylpenicillin and 0 45–0 60 μg/ml for chloramphenicol (Table 1) Similar results were observed for each strain with larger inoculates (10^6 CFU)

The combination with small concentrations of benzylpenicillin usually enhanced the effect of chloramphenicol at concentrations slightly lower than the MIC (Table 2) Small doses of chloramphenicol did not visibly affect the meningococci when combined with benzylpenicillin in concentrations near to MIC However when the chloramphenicol concentration increased a stepwise reduction of the benzylpenicillin concentration that inhibited growth was observed One strain 270 showed indifference with this no antagonistic effect was found

The isobolograms showed no iso additive effect of the two antibiotic drugs and no synergism (Fig 1a and b) Varying degrees of additive effects were observed for each strain except for strain 270 which showed indifference

The additive effect of the two drugs was seen most clearly when moderate amounts of each agent were mixed together (about 50% of the MIC value for each compound) or when low concentrations of benzylpenicillin were combined with higher concentrations of chloramphenicol

TABLE 1 *Minimal Inhibitory Concentrations (μg/ml) of Nine Meningococcal Strains*

Strain	Benzylpenicillin	Chloramphenicol
247	0 018	0 60
270	0 018	0 45
714	0 030	0 45
840	0 026	0 45
837	0 018	0 60
1821	0 018	0 60
3967	0 018	0 60
8884	0 026	0 60
9181	0 022	0 60

TABLE 2 *Modified Carré Method[a] on Agar Strain 247*

	Benzylpenicillin (μg/ml)							
Chloramphenicol (μg/ml)	0 034	0 030	0 026	0 022	0 018	0 014	0 010	0 00
0 70	–	–	–	–	–	–	–	–
0 60	–	–	–	–	–	–	–	–
0 50		–	–	–	–	–	–	+
0 45	–	–	–	–	–	–	–	+
0 40	–	–	–	–	–	–	–	+
0 35		–	–	–	–	–	+	+
0 25	–	–	–		–	–	+	+
0 20	–	–	–	–	–	+	+	+
0 15	–		–	–	–	+	+	+
0 10	–	–	–	–	–	+	+	+
0 05	–	–	–	–	–	+	+	+
0 00	–	–	–	–	–	+	+	+

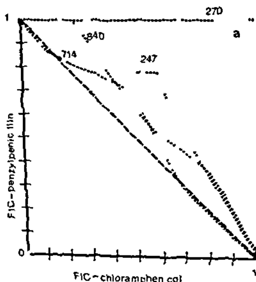
a (ref 4)
– at least 99 % growth reduction
+ growth

DISCUSSION

The action of antibiotics both alone and in combinations may be influenced by a number of factors The effect of protein binding of penicillin is well known and may be eliminated *in vitro* by using a protein free chemically defined medium The adverse effect of agar on antibiotics may be reduced by using washed agar (5)

Against some bacteria benzylpenicillin may have its optimal effect at a certain concentration (9) Our investigation was performed at optimal concentrations of the two antibiotics and also at lower and higher concentrations However since the results were read after 18–24 hours we could not observe the rate of the antibacterial effect during this period

The bacterial density used in this study corre



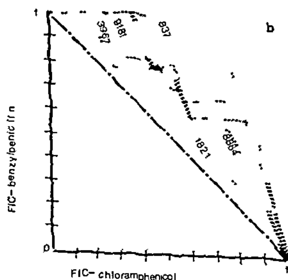


Fig 1a and 1b Isobolograms for nine strains of *N meningitidis* FIC Fractional Inhibitory Conc

i e $\dfrac{\text{Min inhib conc in combination}}{\text{Min inhib conc singly}}$

sponds to that often found in cerebrospinal fluid (CSF) in patients with bacterial meningitis before antibacterial therapy (3) Using megadoses of benzylpenicillin the concentrations of benzylpenicil lin in CSF have been shown in many instances to fluctuate between not detectable and 0 3 µg/ml (6 15) The MIC of meningococci usually varies between 0 02 and 0 3 µg/ml (15) If about 60 per cent of benzylpenicillin reaching the spinal fluid is bound to protein the remaining effect is correspon dingly smaller Chloramphenicol passes readily both normal and pathological meningi

Our *in vitro* investigation showed that low doses of benzylpenicillin seemed not to inhibit the effect of chloramphenicol If this also applies to the *in vivo* situation it must be of particular importance very early or late in the disease when the meningi are almost normal In the normal uninflamed state the meninges present a relatively effective barrier to the entry of the lipid insolubile penicillin molecule In addition there is evidence that penicillin is elimina ted from the CNS by active transport through the chorioid plexus (6) The CSF penicillin concentra tion rises with increments in meningeal inflamma tion (6) Whether this is due to better permeability of the meninges or to inhibition of the transport away from the CNS is at present uncertain

However the proportion of penicillin and chlo ramphenicol changes continuously as a consequence of fluctuations in serum concentration In our investigation concentrations of benzylpenicillin just about the MIC values were indifferent to low doses of chloramphenicol When using this combination in clinical therapy the contrary may be observed viz low concentrations of benzylpenicillin and high concentrations of chloramphenicol This may occur in all poorly accessible tissues

The isobolograms are products of special circum stances during the investigation and strictly spea king are only valid for this situation (2 11 14) If the combinations act indifferently the lines to the point ef equal biological effect may form a quadrate together with the coordinates (Fig 2) The antago nistic effect may be demonstrated when the isobole surpasses this quadrate Two drugs that have an isobole which surpasses this quadrate for one or more combinations may act antagonistically *In vivo* the relative proportions of two drugs change continuously The mixtures may therefore be antagonistic if they show an antagonistic isobolic pattern

In our investigation low doses of chloramphenicol and »optimal« doses of benzylpenicillin seemed to act indifferently while all other combinations of the two drugs seemed to have an additive but not iso additive effect However one strain showed

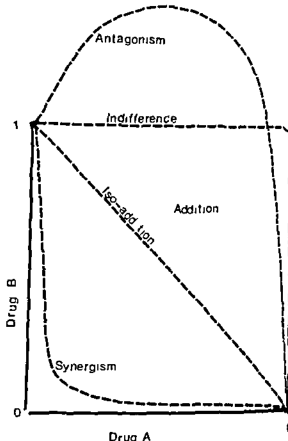


Fig 2 Isobolograms for drug A and drug B showing indifference addition iso addition and synergism

antibiotic indifference in all combinations Whether this combined activity is more bacteriostatic than bactericidal cannot be determined from this study

Jawetz et al (8 9) demonstrated an antagonistic effect of penicillin and chloramphenicol only when the latter was given before or together with benzylpenicillin Chloramphenicol did not interfere with penicillin when it was present either in concentrations below bacteriostatic levels or in high concentrations *Yourassowsky & Monsieur* (17) concluded that the antagonism between chloramp henicol and benzylpenicillin in a liquid medium occurs only with a low concentration of chloramp henicol (0 1–5 µg/ml) However after exposure for 24 hours the results were nearly the same for all combinations In our investigation different combi nations of benzylpenicillin and chloramphenicol did not exhibit antagonism after 18–24 hours but rather some additive effect

We are grateful to Mrs *Grete Myhre* National Institute of Public Health Department of Bacteriology Oslo for excellent techn cal assistance

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ELECTRON MICROSCOPICAL STUDY OF *NEISSERIA MENINGITIDIS* RELEASING VARIOUS AMOUNTS OF FREE ENDOTOXIN

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Andersen B M Skjørten F & Solberg O Electron microscopical study of *Neisseria meningitidis* releasing various amounts of free endotoxin Acta path microbiol scand Sect B 87 109-115 1979

A study has been made of the ultrastructure of four strains of *Neisseria meningitidis* which liberate varying amounts of free endotoxin in a chemically-defined protein free medium The two strains which did not release detectable or only sparse amounts of free filtrable endotoxin were rather uniform in cell size Their cells appeared to be intact and showed a low tendency to aggregate In addition cells of these strains showed only sporadic loose trilaminar membranes and blebs and free membranous structures were sparse in the medium The endotoxin releasing strains liberated a high yield of free structures from the outer cell wall into the medium These structures may represent the lipopolysaccharide (LPS)

Key words *Neisseria meningitidis* ultrastructure free endotoxin

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Gram negative bacteria release endotoxin into the surrounding either as a result of vigorous growth (3) or of special environmental conditions (9) The same also applies to the meningococci (4 10)

In a previous study (1) the liberation of endotoxin during *in vitro* growth of meningococci in a modified protein free medium was found to vary between strains and appeared to differ within the serogroup Maximum endotoxin liberation occurred a few hours after the stationary phase was reached The same pattern of endotoxin liberation was also observed for each strain under other growth conditions

The purpose of this investigation was to study the ultrastructure of four strains which liberate various amounts of free filtrable endotoxin

MATERIAL AND METHODS

Bacterial strains Four isolates were studied three from patients with meningitis (270 714 840) and one from a [illegible]

One strain was serogroup A (714) two were B (270 and 840) and one was Y (247) The strains were subcultured on heated blood agar five or more times before investigation

Medium The medium was prepared as described previously (1 12 13) 9 867 g dehydrated medium 199 with Earles [illegible] nate 3 g dext[illegible] hydrogenpho[illegible] free water to [illegible] 1 M NaOH [illegible] filtration through a pyrogen free prefilter and a membrane filter (1)

Cell growth Precultures were grown for 18 h on heated blood agar at 37°C in 5 per cent CO_2 washed in sterile pyrogen free saline (0 9 per cent NaCl) three times and adjusted to an OD of 0 6 equivalent to a colony count of 10^8–10^9 per ml Optical density was measured by Beckman Model DU 2 Ultraviolet Spectrophotometer wave length 620 nm and slit 0 04 mm

One ml of these suspensions was transferred to 40 ml growth medium The final cultures were grown for 18 h at 37°C in 5 per cent CO_2 spun down at 3000 × g washed and prepared for electron microscopy

Electron microscopy The pellets of bacterial cells were resuspended and fixed in 2 per cent glutaraldehyde in 0 1 M phosphate buffer for 2 h The cells were spun down washed in 0 1 M cacodylate buffer and fixed in 2 per cent osmium tetroxide in the same buffer for 2 h The material was then dehydrated in graded ethanols and embedded in Epon 812

Sections were cut with diamond knives on an LKB Ultrotome III stained with uranyl acetate and lead citrate and examined in a Philips EM 301 S Micrographs were taken at 15 000–45 000 × magnification and enlarged photographically three times

Endotoxin determination Endotoxin determination was made as described previously by the *Limulus* lysate glass slide test procedure (1) All equipment was ascertained to be pyrogen free and all procedures were carried out aseptically

The culture was filtered through sterile non toxic filters (extension set with final filter Travenol code 2C0240) with pore size 0 45 µm The filtrate was stored at −20°C before examination

Endotoxin was also investigated in sonicated samples from the culture (Branson Sonic Power Company Mod D 12 Sonifer cell disruptor) using microtip for 12 min and in centrifugated samples (high speed ultracentrifugation Sorvall 9770 × g at 20°C for 30 min)

RESULTS

Endotoxin determination Free endotoxin was not detected in filtrates of medium after growth of cells of strain 270 and only traces were found in the inoculate used for growth of strain 714 (Table 1) Both strain 247 and 840 liberated free filtrate endotoxin

When sonicating the bacteria high yields of endotoxin were obtained from the crude material of

TABLE 1 *Meningococcal Endotoxin in Filtrates from Cultures of Meningococci Determined by the* Limulus *Lysate Test*

Hours	Strain 247(Y)	270(B)	714(A)	840(B)
0	–	–	–	–
8	1/100[a]	–	–	1/1000
24	1/200	–	–	1/4000

– not detectable endotoxin Endotoxin in the inoculate starting the cultures 247 1/100 270 – 714 1/1 840 1/100

a) Endpoint dilution for positive *Limulus* lysate test

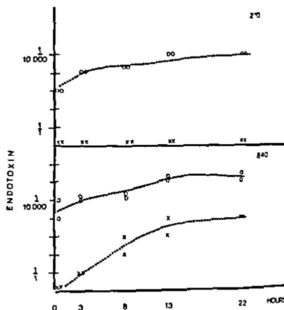


Fig 1 Endotoxin liberated from meningococcal strain 270 and 840 Sonicated crude material o -o and medium which was only filtrated after cell growth x x

all four strains as shown for strain 270 and 840 in Fig 1 Endotoxin was also found in supernatants of centrifuged samples of strain 840 but not of strain 270

Electron microscopy At the time of fixation the viable and direct counts were nearly the same in all the bacterial cell cultures The bacteria of the group B strain (270) which did not liberate endotoxin had the most uniform cell size In contrast to the highly endotoxin releasing group B strain (840) the bacteria were usually intact

All strains studied showed the characteristic multilayered cell envelope of Gram negative bacteria Two trilaminar membranes were evident viz the inner plasma membrane and the outer endotoxin-containing membrane (Fig 2)

Especially in strain 840 the outer trilaminar membrane was often pulled away from the rest of the cell wall or it continued to a neighbouring cell as if the cells had been pulled apart before the cell division had been completed (Fig 3) In some places this outer membrane encompassed the cells forming a ring of cells and membranes This clustering tendency was not found for strain 270 the small cells of which were usually lying distinctly separated from each other A few rodlike trilaminar structures were also found in all groups most frequently in strain 840 (Fig 4) This strain and the other endotoxin releasing strain 247 generally contained membrane fragments blebs and aggregates for free membranes and blebs in the medium (Fig 5a b Fig 6)

2

b

a

3

Fig. 2 The plasma membrane (a) and the outer cell wall (b) is clearly seen on this meningococcus cell of strain 270. Magnification 137 000 ×

Fig. 3 The outer trilaminar membrane continues between neighbouring cells (arrows). Meningococcus strain 840. Magnification 46 000 ×



Fig 4 Rod like trilaminar (arrows) and some tubular structures are present in the medium after growth of cells of meningococcus strain 840 Magnification 46 000 ×

Fig 5 a) Aggregates of blebs and membrane fragments in the medium after growth of cells of meningococcus strain 247 Magnification 46 000 ×

b) Higher magnification showing the trilaminar structure of the blebs A larger ring formed structure consisting of two trilaminated membranes is also present Magnification 137 500 ×

6

7

Fig 6 Blebs, tubular structures and membranes are dispersed in the medium after growth of cells of meningococcus strain 840 Magnification 46 000 ×

Fig 7 Small blebs and tubules are present on the surface of cells of the non-endotoxin liberating strain 270 None of these structures or aggregates of membranes are observed in the growth medium Magnification 46 000 ×

The two trilaminated membranes appeared sporadically in the medium as large, ring-formed structures (Fig 5b) The plasma membrane seemed to disrupt easily when the two trilaminated membranes were separated

Surface blebs of varying size were detected in all specimens In the thin sections of washed bacteria, spherical surface blebs were only sparely distributed, and not all bacteria contained blebs Tubular blebs were found sporadically in all strains, most frequently in the group B meningococci (Fig 7)

The strains that released only sparsely detectable free filtrable endotoxin differed from the endotoxin releasing strains in the following characteristics

a) The cells were more uniform in size were intact and were not generally attached to other cells,
b) Loose trilaminar membranes blebs, aggregates of blebs or other structures were only sporadically detected in the medium

DISCUSSION

DeVoe & Gilchrist (4) observed multiple cell wall blebs on the surface of three strains of *N meningitidis* (group A, B and C) taken form log phase cultures These blebs contained endotoxin However, the cells from the stationary phase cultures showed no surface blebs In our study many bacteria still had surface blebs of varying size even in the stationary phase This was demonstrated in the four strains examined (group A, B B and Y) This difference may perhaps be caused by the use in this study of a chemically-defined, protein free medium since the formation of cell wall components is probably dependent on the growth conditions (4 12)

Tubular blebs could be demonstrated in the group B meningococci and these structures were rather long and numerous Contrary to *DeVoe & Gilchrist* (4) we also found shorter tubular structures in group A and Y meningococci

Blebs attached to the cell wall and free blebs in the medium had a trilaminar structure and seemed to originate from the outer, trilaminar endotoxin containing membrane These cell evaginations are propably not artefacts during preparation since these structures can also be found with negative staining which is a more gentle procedure (7)

Trilaminar structures and blebs are found in the culture supernatants and in the cell envelope fractions (8) *Hill & Weiss* (8) demonstrated large open segmented trilaminar structures from the cell wall enriched fractions containing most endotoxin In our study, the strains releasing free filtrable endotoxin differed from those which did not release endotoxin in that they had substantially more partially free or free fragments of outer trilaminar membranes and blebs and there were aggregates of these membranes and blebs in the medium (Table 2)

This agrees with *Zollinger s* proposal (16) that the outer trilaminar cell wall membrane may represent the lipopolysaccharide (LPS) component with the characteristic properties of bacterial endotoxin

Why only two out of the four strains liberated detectable amounts of free endotoxin requires an explanation Aggregation of LPS gives a large range of particle sizes in the solution (6) However the aggregates observed in the two of the four strains would not be retained on filters with pore size 0 22 µm or larger (15) Neither could affinity to filter membranes as found for other membranes *in vivo* (14) have been the cause When the samples were spun down, endotoxin could still be demonstrated for strain 840 but not for 270 Thus the difference must be real and must correspond to the findings by electron microscopy

The need for different growth requirements might partly explain the varying behaviour of the four strains studied Increased growth rate and aeration may significantly increase the LPS content in the group B meningococci and this has a strain specific variation (5) Perhaps this varying LPS content might explain the different amounts of free

TABLE 2 *Electron Microscopical Characteristics of Four Strains of Meningococci*

Characteristic	Strain 247(Y)	270(B)	714(A)	840(B)
Free membranes	+	–	–	++
Free blebs	++	(+)	(+)	++
Free tubular blebs	(+)	(+)	(+)	++
Aggregates of membranes and blebs	+	–	–	+
Disrupted cells	++	–	(+)	++

– *usually not detected* (+) few + moderate number ++ many

LPS also found in non pathogenic meningococci (10) However, the two group B meningococci studied by us one with free endotoxin and the other with none had almost similar endotoxin content in crude cell material when sonicated

The mechanism of endotoxin liberation may therefore not be related directly to the amount of endotoxin in the outer cell wall Other properties of the bacterial strains, such as cell wall strength osmotic resistance (11) and adhesiveness may play a role as regards endotoxin liberation The difference in tendency to loose cell wall properties can be demonstrated for all groups of meningococci by adding benzylpenicillin to bacteria in an osmotically stable medium (2) Differences in L induction vary within each group of meningococci (2) and may be [illegible]

to another also *in vivo* it could probably explain the different clinical pictures observed in meningococcal diseases

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GENETIC TRANSFORMATION IN *STREPTOCOCCUS SANGUIS*

Spontaneous and Induced Competence of Selected Strains

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Gaustad P Eriksen J & Henriksen S D Genetic transformation in *Streptococcus sanguis* Spontaneous and induced competence of selected strains Acta path microbiol scand Sect B 87 117-122 1979

Nine strains of *Streptococcus sanguis* were examined for competence in genetic transformation with streptomycin resistance (str r) as marker Eight strains belonged to serogroup H and one to the newly described serogroup W Seven of the strains one of which was the reference strain NCTC 7868 (strain Challis) were competent with str r DNA from strain Challis Strains NCTC 9124 (strain Wicky) and 480 were incompetent The efficiency of transformation was examined in four different media Use of Todd Hewitt broth gave frequencies of transformants as high as the more complex media Addition of serum to the transformation media was not essential for the development of competence The presence of a competence factor (CF) in the culture filtrate of strain Challis was confirmed The factor transferred strain Wicky to competence with a great variation in the number of transformants and had no influence on strain 480 On the other hand this spontaneously incompetent strain became competent after addition of culture filtrate from the competent strain 13b in contrast to Wicky which now remained incompetent Thus it is suggested that several factors are involved in the induction of competence of *S sanguis*

Key words *Streptococcus sanguis* competence culture filtrates competence factor

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In a previous paper 95 strains of *Streptococcus sanguis* 90 of which showed spreading zones around the colonies typical of twitching motility were screened for their ability to undergo genetic transformation (3) Seventy two strains 68 of serogroup H and four of the newly-described serogroup W (4) were transformed to streptomycin resistance Spreading zones around the colonies corresponding to twitching motility and fimbriation (5) did not seem to be a requirement for competence (3)

The term competence is defined as the ability to undergo transformation by homologous DNA The physiological state of competence seems to be connected with the mechanism of DNA penetration through the cell wall (2 14)

Pakula and coworkers (11, 12) reported that sterile filtrates from cultures of a competent streptococcus serogroup H strain Challis contain a competence factor (CF) which induces competence in a non transformable strain Wicky of the same serological group

The purpose of this paper is to examine the requirement for soluble factors in the transformation of several strains of *S sanguis*

MATERIAL AND METHODS

S sanguis serogroup H strains 13b 480 10115a 10140a and 14506 and serogroup W strain 12318 were among those used in a previous study (3) All these strains showed spreading typical of twitching motility Three reference strains were obtained from the National

Collection of Type Cultures London England NCTC 7868 (strain Challis) NCTC 9124 (strain Wicky) and NCTC 7863 (Washburn type I) Strain NCTC 7863 showed spreading

Media The strains were maintained on 5% human blood agar plates incubated in a humid atmosphere (5) Precious transformation studies have mostly been carried out in rather complex media (6 7) The transformation medium used in our experiments was Todd Hewitt Broth (TH) from Oxoid Ltd London Three other media were also used ET3 and ST described by *Pakula & Walczak* (12) and PYG described by *Perry & Slade* (13)

DNA DNA was isolated from streptomycin resistant (str r) mutants of a competent substrain of strain Challis obtained from Dr *Pakula* Toronto Canada and prepared as described previously (3)

Transformation procedure The transformation experiments were carried out as described by *Henriksen & Eriksen* (Method no 1) with some minor alterations (3) Eighteen h cultures in TH were used One tenth ml of culture in 1 8 ml TH contained a suitable number of bacteria (13×10^7/ml) The media were prewarmed and not shaken during cultivation Optical dencity of 18 h cultures seemed to be quite constant for a given strain The final concentration of str r DNA in the experiments was 20 µg/ml This amount produced maximal yield of transformants which was tested for each str r DNA used

The transformation procedure was carried out as follows in triplicate All incubations were at 37° C One tenth ml of an 18 h culture was inoculated into 1 8 ml TH and incubated for 90 min to reach the stage of optimal competence Strain Challis presents at that time the early exponential phase of growth One tenth ml of str r DNA was added (final concentration of str r DNA was 20 µg/ml) The culture grew for another 120 min to express the phenotype of the marker DNA se was not added Thus the transformation method used is semiquantitative and not designed for exact calculation of the transformation frequency Growth was stopped on ice bath Aliquots of 0 1 ml of suitable dilutions were spread on blood agar plates containing 100 µg/ml streptomycin and on plain blood agar plates The plates were incubated for 48 h and colony counts were performed The number of transformants was obtained from the streptomycin plates Counting of blood agar plates was made to check that str r DNA itself did not inhibit the growth of the bacteria and to calculate the frequency of transformants The saline control was carried out using the same main procedure and 0 1 ml physiological saline and no str r DNA was added The saline control ought to be identical with the blood agar control if no inhibition occurs To detect spontaneous streptomycin resistant mutants one tenth ml of the undiluted culture was spread on spreptomycin plates The number of bacteria/ml at the beginning of the experiment (growth stopped on ice bath) was used for calculation of the rate of growth

In addition transformation of strains Challis Wicky and 10115a was tested using a modification of the quantitative technique described by *Bøvre* (1) In these experiments the procedure described above was maintained except that pancreatic DNA se (50 µg/ml) was added at the end of the 120 min incubation period and then 0 1 ml aliquots of suitable dilutions were spread on blood agar plates The plates were incubated for 6 h to allow full phenotypic expression and transferred to a layer of streptomycin Brain Heart Infusion agar (Difco) The final concentration of streptomycin of the plates was 100 µg/ml

Culture filtrates Culture filtrates were prepared by a 10% inoculate of an 18 h TH culture on prewarmed TH containing 2 5% inactivated horse serum The culture was incubated at 37° C for 3 h Growth was stopped on ice bath and the culture was then centrifuged at 4°C for 20 min at 5500 × *g* The supernatant was sterilized by filtration through Millipore filter 0 45 µm Testing was made for sterility The culture filtrate was tested in transformation divided into suitable volumes and kept frozen at −20° C for later use

The use of culture filtrates was adapted by a modification of the main transformation procedure [illegible]

Growth rate The ratio of colony forming units (CFU) [illegible] Challis and [illegible]

0 to 3 h Suitable dilutions were inoculated on blood agar plates and CFU/ml was calculated after growth for 48 h The examinations were made in triplicate

The protein content was determined according to Folin Ciocaltau (8) in parallel samples Growth was stopped by heating in boiling water bath for 30 min The samples were centrifuged and the supernatant was removed The cells were washed once with cold saline and centrifuged and then examined for protein content

Samples for microscopic examination were removed from the TH culture at different intervals One or two drops were removed gently to avoid breaking the streptococcal chains Gram staining was carried out in the usual way

RESULTS AND DISCUSSION

In order to establish conditions for semiquantitative transformation experiments with the recent isolates of *S sanguis* serogroup H and W preliminary tests were carried out with strain Challis It was assumed that the conditions suitable for that strain could be adopted for other strains

Four different media one of which was TH with 5% horse serum were compared The presence of serum in the media for production of CF and transformation has been reported to be of importance (12) The results in Table 1 indicate that ST and TH both with serum gave superior and

TABLE 1 *Efficiency of Transformation of Strain Challis in Four Different Media*

Medium with 5% serum	CFU/ml at start	CFU/ml at end of DNA exposure	Transformants/ml as CFU at end of DNA exposure	Frequency of transformants in %
ET 3	3.55×10^7	9.4×10^7	1×10^4	0.01
ST	13.5×10^7	107×10^7	1.85×10^7	1.7
PYG	1.85×10^7	8×10^7	2.5×10^5	0.3
TH	13.1×10^7	133×10^7	2.4×10^7	1.8

One tenth ml of an 18 h culture was inoculated into 1.8 ml medium and counted (start value column 2) Inoculation [illegible] ET 12 and PYG described by *Holdeman* & *Moore* (13)

practically equal results A second experiment was carried out in TH without serum using the main procedure as before but changing the time from start of growth to addition of str r DNA from 0 to 180 min After addition of DNA growth was allowed for 120 min The transformation efficiencies are shown in Fig 1 These indicate that addition of serum to the media is not required for the production of CF and development of competence (10) TH is a suitable medium and transformation occurred irrespective of whether serum was present or not It would appear that [illegible]

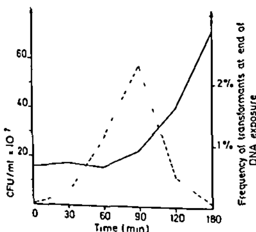


Fig 1 Transformation of [illegible] added at different [illegible] was [illegible] continuous line) is based on counts of colony forming units (CFU) at the start of exposure to DNA in each case

before addition of DNA produces optimal transformation efficiency of strain Challis This corresponds to the early exponential phase of growth

Strain Challis had a much higher frequency of transformants than the recent isolates (strains 13b 10115a 10140a 12318 and 14506) In strain Wicky 2×10^{-5}% of the bacteria exposed to str r DNA were transformed This strain has been considered by other authors to be incompetent or poorly transformable (9) due to the few transformants The use of a more sensitive technique (1) did not increase the number of transformants Accordingly strains with transformation frequency less than 3×10^{-5}% are considered to be incompetent Strain 480 one of the recent isolates had no detectable transformation (Table 2) Variation of the time before addition of str r DNA was examined for strain 480 but no transformants were detected One explanation for the much higher frequency of transformants in strain Challis may be the use of autologous DNA Transformation studies of strain Wicky with autologous DNA did not increase the number of transformants However, experimental conditions optimal for strain Challis may still not necessarily be equally suitable for the other strains

In accordance with earlier investigations (9, 12) CF could be demonstrated in culture filtrates of strain Challis (Table 3) No attempt was made to

isolate the factor The factor transferred strain Wicky to competence The frequency of transformants varied in the range of 1 9 − 10 8 %

The use of the technique described by *Bovre* (1), permitting full phenotypic expression of the transformants, did not change significantly the number of transformants for strain Challis and strain Wicky with filtrate of strain Challis Thus the phenotypic expression seemed to be complete for these strains in the main experimental procedure On the other hand, using the technique of *Bovre* strain 10115a had a 25 times larger number of transformants This indicates that the conditions in the main procedure are not always optimal

TABLE 2 *Transformation of* Streptococcus sanguis *Eight Strains of Group H and one of the Newly described Group W*

Recipient strain	CFU*/ml at end of DNA§ exposure	Transformants CFU/ml	Frequency %
NCTC 7868 Challis	344 $\times 10^7$	5 8 $\times 10^7$	1 6
	229 $\times 10^7$	2 57 $\times 10^7$	1 1
	275 $\times 10^7$	6 3 $\times 10^7$	2 3
	233 $\times 10^7$	2 9 $\times 10^7$	1 2
NCTC 7863	20 9 $\times 10^7$	512000	0 2
	22 9 $\times 10^7$	247000	0 1
	9 3 $\times 10^7$	10800	0 01
13b	13 1 $\times 10^7$	430000	0 3
10115a	62 4 $\times 10^7$	300	0 0005
10140a	47 3 $\times 10^7$	4500	0 001
14506	54 8 $\times 10^7$	34400	0 006
12318**	37 3 $\times 10^7$	23000	0 006
NCTC 9124 Wicky	33 $\times 10^7$	60	2×10^{-5}
480	16 8 $\times 10^7$	0	$<6 \times 10^{-7}$

Procedure for semiquantitative streptomycin resistance transformation in Todd Hewitt broth (TH) is described in legend to Table 1 and in text

* CFU = colony forming units

** Strain belonging to serogroup W

§ Str r DNA derived from strain Wicky in all other experiments str r DNA of strain Challis was used

TABLE 3 *Influence of Filtrate from Competent Strains on the Frequency of Transformation of the Strains Wicky and 480*

Recipient strain	Frequency of transformants		
	with filtrate from strain Challis	with filtrate from strain 13b	without filtrate
NCTC 9124 Wicky	1 9 − 10 8 %	$<3 \times 10^{-5}$ %	$<3 \times 10^{-5}$ %
480	$<3 \times 10^{-5}$ %	0 15 %	$<3 \times 10^{-5}$ %

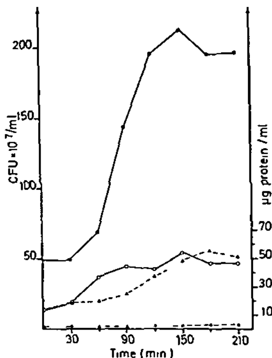
Procedure for semiquantitative streptomycin resistance *transformation in Todd Hewitt broth* (TH) is described in legend to Table 1 and in text In all experiments str r DNA from strain Challis was used

The ratio of CFU to protein per ml was examined and related to the number of bacteria per chain in cultures of strains Challis and Wicky These two strains grew quite differently in TH broth While Challis grew as an even suspension Wicky showed a clear supernatant over a heavy precipitate of bacterial cells During growth for 3 h there was very little increase in the CFU for Wicky as compared with Challis On the other hand the production of protein was nearly the same for both strains during the same period (Fig 2)

Microscopic at examination of the number of bacteria in the chains from growth in TH broth showed great differences For strain Challis the number of bacteria in the chain varied between 5 and 15 while Wicky mostly showed chains consisting of 40 – 100 bacteria This may indicate that the long chains of Wicky result in less CFU in spite of the same rate of growth measured by content of protein per ml The great variations in the transformation frequencies with filtrate Challis may thus be explained for this strain The possible effect of filtrate Challis on the chain length of strain Wicky has not been studied in detail Other strains with more constant and fewer cells in a chain showed a more constant number of transformants

The culture filtrate of strain Challis was added to a TH culture of the incompetent strain 480 to examine whether this strain also could be transformed to competence but no transformation occurred. The culture filtrate of strain Challis is not generally active Another competent strain 13b a recent isolate was also examined for CF production This filtrate however did not induce competence in Wicky On the other hand strain 480 became competent (Table 3) Thus the two filtrates and the two recipient strains showed different properties which suggests that several factors may be involved in the induction of competence of *S sanguis* Among the recent isolates spreading zones around the colonies and competence (spontaneous or induced) were present while spreading was present only in one of the competent reference strains namely NCTC 7863 As mentioned by *Henriksen & Eriksen* spreading is not a prerequiste for competence in *S sanguis* contrary to what has been shown in some species of *Neisseraceae* (3) However a connection between competence of the recent isolates and the spreading character has not been ruled out

In transformation studies of *S sanguis* strains Challis and Wicky have mainly been in focus (9 12) both for the mechanism of competence and for the properties of CF The finding of an active factor in culture filtrate from another strain of *S sanguis* than Challis acting on a different recipient opens



ml TH
●—● Growth rate of strain Challis CFU/ml
O—O Protein per ml strain Challis
▲__▲ Growth rate of strain Wicky CFU/ml
△__△ Protein per ml strain Wicky

new aspects in transformation studies The next step in this investigation might therefore be comparison of the filtrates from additional competent and incompetent strains among the recent isolates of *S sanguis* from the point of view of their ability to induce competence in different strains

The excellent technical assistance of Mrs *Eva Løvstad* and Mrs *Aud Stendahl* is gratefully acknowledged

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Acta path microbiol scand Sect B 87 123–128 1979

GENETIC TRANSFORMATION IN *STREPTOCOCCUS SANGUIS*

Distribution of Competence and Competence Factors in a Collection of Strains

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Gaustad P Genetic transformation in *Streptococcus sanguis* Distribution of competence and competence factors in a collection of strains Acta path microbiol scand Sect B 87 123–128 1979

Forty-one strains of *Streptococcus sanguis* (37 of serogroup H and four of the newly-described serogroup W) were examined semiquantitatively for genetic transformation with streptomycin as marker The material comprised eight reference laboratory strains and 33 recent isolates Eighteen strains (16 of serogroup H and two of W) showed spontaneous competence in genetic transformation (without added competence factor i e culture filtrate) Individual culture filtrates from 19 spontaneously competent and ten incompetent strains were tested for competence inducing effect on 23 spontaneously incompetent strains Competence was induced in 16 of the strains and 20 of the culture filtrates were active There was considerable variation with respect to the number of recipient strains which were induced to competence by individual filtrates Furthermore the recipients varied as regards the number of filtrates that were able to induce that particular strain There was some relationship but no complete association between competence competence inducing ability and the occurrence of spreading zones around the colonies assumed to correspond generally to fimbriation Thus three incompetent strains had an active culture filtrate and one spontaneously competent strain had an inactive filtrate Most but not all strains with spontaneous or inducible competence showed spreading as did most of the strains from which broadly inducing filtrates could be produced

Key words *Streptococcus sanguis* competence in genetic transformation culture filtrates competence factor spreading colonies

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Received 12 vii 78 Accepted 31 x 78

Previous studies on genetic transformation of streptococci have concentrated mostly on a few strains in particular the *Streptococcus sanguis* (serogroup H) strain Challis (NCTC 7868) and strain Wicky (NCTC 9124) Strain Challis has been shown to produce a competence factor (CF) which causes the otherwise incompetent strain Wicky to acquire competence (4 13)

The isolation of a number of *S sanguis* strains characterized by the production of spreading zones around the colonies caused by twitching motility and presumably due to fimbriation (9 10) suggested the necessity for further transformation studies to include these strains *Henriksen & Eriksen* (7) screened 95 strains of *S sanguis* mostly recent isolates from the human throat for competance in transformation using DNA from a streptomycin resistant mutant of strain Challis Seventy three strains were competent Three of the incompetent strains were tested with autologous DNA and two were transformed

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transformants per ml) or negative (less than 10^2 transformants per ml) This is in accordance with earlier reports based on transformation of the incompetent strain Wicky (6 12) DNA se was not added to terminate DNA exposure The transformation method is thus considered to be semiquantitative (6) Controls without addition of filtrate and str r DNA as well as transformation of strain Challis were included In some control experiments the method of *Bøvre* (1) which allows phenotypic expression also of those cells transformed very late during the 120 min DNA exposure was used (6) All incubations were performed at 37°C

RESULTS AND DISCUSSION

Forty-one strains of *S sanguis* (serogroup H and W) were examined by genetic transformation with streptomycin as marker Eight reference laboratory strains and 33 recent isolates were included Transformation experiments with str r DNA from strain Challis gave spontaneous transformation (without added CF i e culture filtrate) in 18 of the strains (Table 1)

A close relationship has been reported between competence in transformation and fimbriation in some strains of *Neisseriaceae* (2 5) Among the strains of *S sanguis* there was no clear relationship between spontaneous competence and the occurrence of spreading zones around the colonies presumably corresponding to twitching motility and fimbriation (9) However most spontaneously competent strains did possess spreading zones (Table 1) especially the recent isolates

Individual [illegible]

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There was considerable variation in the number of recipient strains induced to competence by individual filtrates The recipients also varied with regard to the number of filtrates able to induce competence (Table 2) The maximum number of recipients induced by an active filtrate was 13 the minimum number one The maximum number of filtrates active on one recipient was 16 the lowest number one (Table 2) The results indicate a general difference between the recent isolates and the reference strains Filtrates from 15 of the recent isolates induced competence in 6 to 13 recent isolates but not in any of the reference strains Use of culture filtrates from the reference strains Challis Blackburn and Channon gave only transformation of strain Wicky as shown by others (11 13) The remaining 22 spontaneously incompetent strains were not induced to competence by these culture filtrates

The finding that three incompetent strains viz Blackburn Channon and 14567 had filtrates which induced competence may indicate either that the methods used to screen for transformation are not sufficiently sensitive or that the strains lack one or more of the factors necessary for transformation but have other factor(s) sufficient for the induction when the former factor(s) is provided The possible existence of several factors in the transformation process and both a CF and an inhibition factor (IF) [illegible]

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Filtrate from the spontaneously competent strain NCTC 7865 only produced competence in strain ATCC 10558 which actually is a spontaneously incompetent substrain of the same original strain Washburn type I/II (14) but obtained from a different culture collection It is conceivable that during subcultivation ATCC 10558 has lost some factor(s) necessary for competence Since neither strain NCTC 7865 nor strain ATCC 10558 produced spreading colonies typical of twitching motility their difference in competence was apparently not related to those characteristics

Pakula & Walczak (13) were able to transform strain Washburn type I/II spontaneously and to produce an active culture filtrate using medium ET3 with serum Without serum the strain was non transformable spontaneously but the filtrate gave transformation of the strain itself In our experiments with TH the two substrains of Washburn type I/II had quite different characteristics in transformation This indicates that the conditions optimal for transformation may vary from medium

In the previous report on competence in transformation of nine strains of *S sanguis* (6) belonging to serogroup H and the newly-described serogroup W (8) it was suggested that different factors may be involved in genetic transformation of *S sanguis* these factors being related both to recipient strains and source of CF

The intention of the present study was to examine further the different factors of importance for the induction of competence by testing the activity of CFs from numerous strains on a large number of spontaneously incompetent *S sanguis* strains

MATERIALS AND METHODS

The strains of *S sanguis* included were isolates studied by *Henriksen* and coworkers (7 9 10) and reference strains from the National Collection of Type Cultures (NCTC) London and the American Type Culture Collection (ATCC) Rockvill MI The strains used in the transformation experiments and for culture filtrates are listed in Table 1 Strains Blackburn (NCTC 10231) and Channon (NCTC 7869) were not included in the previous studies by *Henriksen* and coworkers

The study of spreading zones around the colonies on blood agar plates was performed as described by *Henriksen & Henrichsen* (9)

Methods for selection of streptomycin resistant mutants and production of DNA have been described (7) DNA from streptomycin resistant mutants (str r) of strain Challis was used Strains incompetent with this DNA were also tested with autologous str r DNA

For production of culture filtrates the strain in question was cultivated in Todd Hewitt broth (TH) with 2 5% inactivated horse serum as described previously (6) The transformation experiments using culture filtrates were carried out in TH Previous studies indicated that incubation of the culture for 60 min before addition of the filtrate *i e* CF is suitable for transformation of strain Wicky (6) and that incubation time was maintained for the other strains examined Str r DNA (20 µg/ml) was added after exposure to CF for 30 min Incubation with DNA was continued for another 120 min to allow phenotypic expression before cultivation on blood agar plates with or without 100 µg/ml streptomycin The results were read after incubation for two days and were recorded as positive (more than 10

TABLE 1 *Strains of* Streptococcus sanguis *Spontaneous Competence and Formation of Spreading Zones around the Colonies*

Strain[a])	Serogroup	Spontaneous competence in transformation[b])	Spreading zones around the colonies[c])
NCTC 7868 Challis	H	+	-
NCTC 7863	H	+	+
NCTC 7865	H	+	-
ATCC 10556	H	+	+
2b	H	+	+
10a	H	+	+
13b	H	+	+
10115a	H	+	+
10140a	H	+	+
10152a	H	+	+
10227a	H	+	+
10229c	H	+	+
10336b	W	+	+
10442b	H	+	+
10543b	H	+	+
10738a	H	+	+
12318	W	+	+
14506	H	+	+
NCTC 7869 Channon	H	-	-
NCTC 9124 Wicky	H	-	-
NCTC 10231 Blackburn	H	-	-
ATCC 10558	H	-	-
445	H	-	+
480	H	-	+
2908	H	-	+
10043a	W	-	+
10067	H	-	+
10194a	H	-	+
10279c	H	-	+
10281b	W	-	+
10299b	H	-	+
10305	H	-	+
10414b	H	-	+
10560c	H	-	+
10780b	H	-	+
10784b	H	-	+
10790	H	-	+
10807	H	-	+
13843	H	-	+
14558	H	-	+
14567	H	-	+

a) ATCC = American Type Culture Collection Maryland USA
NCTC - National Collection of Type Cultures London England
Strains without type culture collection designation were isolated by and received from Dr *S D* [illegible]
letters following the isolation number

b) Competence in genetic transformation with DNA from a streptomycin resistant mutant of strain Challis and without the use of culture filtrate Procedure as described in ref 6 (main method)

c) See ref 9

transformants per ml) or negative (less than 10[2] transformants per ml) This is in accordance with earlier reports based on transformation of the incompetent strain Wicky (6 12) DNA se was not added to terminate DNA exposure. The transformation method is thus cons dered to be semiquantitative (6) Controls without add tion of f ltrate and str r DNA as well as transformation of strain Challis were included In some control experiments the method of *Bøvre* (1) wh ch allows phenotyp c express on also of those cells transfor med very late during the 120 min DNA exposure was used (6) All incubations were performed at 37°C

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Individual culture filtrates from 18 spontaneous *ly competent* and 11 incompetent strains (with str r DNA [illegible]

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to medium and with enrichment of the medium Strains Challis and Wicky have been used as reference strains in our transformation experiments concerning cultivation conditions and the definition of competence in transformation Strain Wicky is considered to be an incompetent or poorly transformable strain, and has a transformation frequency of 2×10^{-5} per cent which corresponds to less than 10^2 transformants per ml culture In most cases, the strains registered as competent yield more than 10^4 transformants per ml, corresponding to a percentage of transformants of 3×10^{-3}% or more It is possible that the conditions optimal for the transformation process may vary from one strain to another, especially if production of several factors is necessary, and that strains incompetent in this study may become competent when using other experimental and recording conditions Use of a technique

TABLE 2 *Induction of Competence in Spontaneously Incompetent* Streptococcus sanguis *by Culture Filtrates*[a])

Recipient *strain*[b]) / Source of filtrates[c])	NCTC 7868, Challis	13b	NCTC 7863	2b	10a	10115a	10140a	10152a	10227a	10229a	10336b	10442b	10543b	10738a	12318	14506	14567	NCTC 7865	NCTC 10231, Blackburn	NCTC 7869 Channon	NCTC 9124 Wicky	ATCC 10556	ACTT 10558	445	480	10279c	10299b	10790
445	–	+	+	+	+	+	–	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–
480	–	+	+	+	+	+	–	–	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–
2908	–	+	+	+	+	+	–	–	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–
10067	–	+	–	+	–	+	–	–	–	–	+	+	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–
10194a	–	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–
10279c	–	+	+	+	+	+	–	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–
10281b	–	+	+	+	+	+	+	–	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–
10305	–	+	+	–	+	+	+	+	+	+	+	+	+	+	+	–	+	–	–	–	–	–	–	–	–	–	–	–
10560b	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–
10780b	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–
10807	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–
13843	–	+	+	+	+	+	–	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–
14558	–	+	+	–	+	–	–	–	+	+	+	+	+	+	+	–	+	–	–	–	–	–	–	–	–	–	–	–
10299b	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–
ATCC 10558	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–
NCTC 9124 Wicky	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+	–	–	–	–	–	–	–	–
NCTC 10231 Blackburn	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
NCTC 7869 Channon	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
10043a	–	–	–	–	–	–	–	–	–	–	–	–	–		–	–	–	–	–	–	–	–	–	–	–	–	–	–
10514b	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
10784b	–	–	–	–	–	–	–	–	–	–	–	–	–		–	–	–	–	–	–	–	–	–	–	–	–	–	–
10790	–	–	–	–	–	–	–	–	–	–		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
14567	–	–	–	–	–	–	–	–	–	–		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

a) Semiquantitative transformation with streptomycin resistant (str r) mutant DNA from strain Challis was performed with the respective culture filtrates added (ref 6 and text) With strains 14558 and 14567 control experiments were performed by quantitative str r transformation (refs 1 6 and text) The seven strains found incompetent with any filtrate were retested using autologous str r DNA (from mutant of the same strain) and culture filtrates from the strains Challis and 13b see text for these additional results

b) Strains not transformed to streptomycin resistance without the use of culture filtrate (spontaneously incompetent) (see Table 1)

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\+ induction of competence (see text)

– no induction of competence

which permits full phenotypic expression of the genetic marker (1) in experiments with strains 14558 and 14567 did not reveal competence induction by any filtrate which was inactive with the screening technique However the number of transformants obtained increased in some cases

The two spontaneously competent strains ATCC 10556 and NCTC 7863 are substrains of Washburn type I (14) The filtrates from the two strains had different characteristics Filtrate NCTC 7863 was the only reference strain that converted 12 recent isolates of *S sanguis* to competence while filtrate ATCC 10556 did not induce competence in any strain in this study (Table 2) A dissociation of the characteristics competence and production of active filtrate is indicated by these findings

Generally there seemed to be relationship between the simultaneous occurrence of spontaneous competence and spreading colonies on the one hand and the ability to produce filtrates inducing competence in many strains on the other Atypical is strain 14567 which is incompetent but which has zones and which induced competence in 13 strains On the other hand strain ATCC 10556 which was competent with zones had no competence inducing power Strain Challis which was competent without zones induced competence in strain Wicky only as did the two incompetent non spreading strains Blackburn and Channon The competent non spreading strain NCTC 7865 induced competence only in strain ATCC 10558 which is a subculture of the same strain

All spontaneously incompetent strains which were induced to competence by filtrates of many strains were of the type which had spreading zones around the colonies The non spreading strain Wicky was induced by filtrates of strains Challis Blackburn and Channon only and the non spreading strain ATCC 10558 only by the closely related strain NCTC 7865 (see above) However the spreading strain 10299b was induced to competence by two filtrates only (from strains 10227a and 14567) and several spreading strains were not induceable by any filtrate

Despite the exceptions observed the results generally indicate a relationship between the appearance of spreading zones the production of competence inducing culture filtrate and competence (including the ability to become induced to competence by filtrate) If it is proved that such a relationship exists when critically examined in future experiments it must be complex and will include unknown additional variables

The excellent technical assistance of Mrs *Eva Løvstad* is gratefully acknowledged

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to medium and with enrichment of the medium Strains Challis and Wicky have been used as reference strains in our transformation experiments concerning cultivation conditions and the definition of competence in transformation Strain Wicky is considered to be an incompetent or poorly transformable strain and has a transformation frequency of 2×10^{-5} per cent which corresponds to less than 10^2 transformants per ml culture In most cases the strains registered as competent yield more than 10^4 transformants per ml corresponding to a percentage of transformants of 3×10^{-3}% or more It is possible that the conditions optimal for the transformation process may vary from one strain to another, especially if production of several factors is necessary, and that strains incompetent in this study may become competent when using other experimental and recording conditions Use of a technique

TABLE 2 *Induction of Competence in Spontaneously Incompetent* Streptococcus sanguis *by Culture Filtrates*[a])

Recipient strain[b])	*Source of filtrates*[c])	NCTC 7868 Challis	13b	*NCTC 7863*	2b	10a	10115a	10140a	10152a	10227a	10229a	10336b	10442b	10543b	10738a	12318	14506	14567	NCTC 7865	NCTC 10231 Blackburn	NCTC 7869 Channon	NCTC 9124 Wicky	ATCC 10556	ACTT 10558	445	*480*	10279c	10299b	10790	1080[illegible]
445		–	+	+	+	+	+	–	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	
480		–	+	+	+	+	+	–	–	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	
908		–	+	+	+	+	+	–	–	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	
10067		–	+	–	+	–	+	–	–	–	–	+	+	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	
10194a		–	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	
10279c		–	+	+	+	+	+	–	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	
10281b		–	+	+	+	+	+	+	–	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	
10305		–	+	+	–	+	+	+	+	+	+	+	+	+	+	+	–	+	–	–	–	–	–	–	–	–	–	–	–	
10560b		–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	
10780b		–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	
10807		–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	
13843		–	+	+	+	+	+	–	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
14558		–	+	+	–	+	–	–	–	+	+	+	+	+	+	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–
10299b		–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	
ATCC 10558		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–
NCTC 9124 Wicky		+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+	–	–	–	–	–	–	–	–	–
NCTC 10231 Blackburn		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
NCTC 7869 Channon		–	–	–	–	–	–	–	–	–	–	–		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
0043a		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–		–	–	–	–	–	–	–	–	–
10514b		–	–	–	–	–		–	–	–	–	–	–	–	–	–	–	–	–	–		–	–	–	–	–	–	–	–	–
0784b		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
0790		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
14567		–	–	–	–	–	–	–	–		–	–	–	–	–	–	–	–	–	–		–	–	–	–	–	–	–	–	–

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+ induction of competence (see text)

– no induction of competence

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AFFINITY CHROMATOGRAPHY OF INFLUENZA VIRUS ON IMMOBILIZED α- AND β-KETOSIDES OF NEURAMINIC ACID DERIVATIVES

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Holmquist L. & Nilsson G Affinity chromatography of influenza virus on immobilized α and β-ketosides of neuraminic acid derivatives Acta path microbiol scand Sect B 87 129–135 1979

In order to purify influenza viruses which contain neuraminidase as a constituent of the virus-coat surface a study was made of the affinity of formaldehyde inactivated viruses for immobilized ketosides of neuraminic acid derivatives Attempted desorption of viruses bound to columns of the conjugates was performed by addition of the benzyl α ketoside of *N* acetylneuraminic acid to the eluent The conjugates with α ketosidically bound neuraminic acids adsorbed virus which could be desorbed with the enzyme substrate When β ketosidically immobilized *N* acetylneuraminic acid was saturated with virus and the conjugate was washed with buffer until it was free from neuraminidase activity no more virus could be desorbed with neuraminidase substrate-containing eluents However addition of substrate to the buffer during the washing procedure resulted in a neuraminidase activity peak in the effluent All conjugates bound firmly a portion of the viruses which remained on the sorbents even after excessive treatment with eluent containing the neuraminidase substrate When the conjugates were saturated with virus all sites which bound the viruses strongly were blocked and the remaining binding sites could be utilized for reversible adsorption of the viruses Using this method crude influenza virus vaccine could be separated into allantoic fluid components and pure virus Centrifugation of the virus preparation in a sucrose gradient indicated that this column procedure did not solubilize the viral neuraminidase Using freshly prepared non inactivated influenza virus the portion of gel bound viruses which could not be desorbed by neuraminidase substrate was diminished

Key words Influenza virus affinity chromatography immobilized ketosides neuraminic acid derivatives

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Our studies on neuraminidase (glycoprotein *N* acetylneuraminylhydrolase EC 3 2 1 18) show that the enzyme released by *Vibrio cholerae* into culture media is reversibly adsorbed on α *N* acetylneuraminic acid Sepharose (3) The enzyme may be desorbed from the matrix by adding the benzyl α ketoside of *N* acetylneuraminic acid a low molecular weight neuraminidase substrate to the eluent As neuraminidase is a constituent of the influenza virus-coat surface a study was made of the affinity of influenza virus for immobilized ketosides of neuraminic acid derivatives

It was predicted that reversible adsorption of influenza virus on matrix-bound neuraminic acid derivatives could be used for specific purification of the virus

MATERIAL AND METHODS

All virus preparations used except one were formaldehyde inactivated and were obtained from the National Bacteriological Labor[illegible]

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MATERIAL AND METHODS

All virus preparations used except one were formaldehyde inactivated and were obtained from the National Bacteriological Laboratory Stockholm Sweden Vaccines against the influenza virus type A/Dunedin/4/73 or type A/Victoria/3/75 and type B/Hongkong/8/73 purified by differential centrifugation and crude preparations of influenza virus in allantoic fluid contained 8192 and 1024 arbitrary haemagglutinating units per ml respectively One experiment was performed with a crude preparation of non inactivated influenza virus Victoria strain

General Methods

Synthesis and purification of neuraminic acid derivatives were followed by TLC the general methods used have been described previously (3 4)

The Sepharose 4B conjugates of neuraminic acid derivatives were prepared according to (3) using the 2 aminoethylaminocarbonylmethyl ketosides of the non ulosonic acids and the conditions for preparation of the type V conjugate The 7 C analogues of the different conjugates were prepared according to (3)

The amount of ketosidically bound neuraminic acid derivatives was in the range of 2–3 µmol/ml wet gel and 1–2 µmol/ml wet gel for the α and β ketosides respectively as determined according to (3 5)

Peracetylated *N* propionylneuraminic acid was synthesized as described by *Meindl & Tuppy* (7)

Affinity column experiments were performed at 4° C unless otherwise stated

The Lowry technique (2) was used for protein determination with bovine serum albumin as protein standard

Sedimentation Experiments

Virus suspension (1 ml) in 0 15 M sodium chloride or 0 05 M Tris maleate buffer pH 6 4 was layered onto a linear sucrose gradient and centrifuged in the Beckman SW 40 rotor at 12 000 rev/min for 120 min as described by *Laver & Webster* for purification of influenza virus (6) The tubes (9 5 × 1 5 cm) contained 12 5 ml of 5–20% Sucrose in 0 15 M sodium chloride After completed centrifugation fractions (1 ml) were collected through a hole at the bottom just where the tube walls bend The fraction remaining in the bottom (ca 0 5 ml) was collected and diluted with buffer to 1 ml

By including blue dextrane in the bottom fraction it could be demonstrated that it did not mix with the other fractions taken from the tube

One diffuse band at the top one in the middle and one at the bottom of the tube could be seen on centrifugation of concentrated virus suspension By slicing the tube the same neuraminidase activity pattern was obtained as with the puncture technique

After dialysis of the fractions against 0 1 M sodium acetate pH 5 5 or 0 1 M Tris maleate buffer pH 6 4 both 0 01 M as regards calcium chloride the protein concentration and neuraminidase activity were determined The concentration of benzyl α ketoside of *N* acetylneuraminic acid was 400 µg/ml and incubation was performed at 37° C Aliquots of 50–200 µl were removed and analyzed for *N* acetylneuraminic acid (8)

Haemagglutination Test

Haemagglutination titrations were performed in Takatsy plates by adding 50 µl of stepwise double dilutions of virus samples in phosphate buffered saline to 50 µl of 0 3% chicken erythrocytes The number of haemagglutinating (HA) units per ml of the sample is expressed as the reciprocal of the last dilution giving complete agglutination

Syntheses

Butoxycarbonylmethyl α ketoside of tetra – 0 – acetyl N propionylneuraminic acid Peracetylated *N* propionylneuraminic acid (900 mg) was transformed to its 2 chloro derivative according to (7) and shaken with silver carbonate (1g) pulverized Drierite (2 g) and butyl glycolate (20 ml) overnight The mixture was filtered and solids were washed with acetone water (2 1 v/v) The combined filtrate and washings were passed slowly through a Dowex 1 X 4 column (acetate form 2 × 15 cm) equilibrated with acetone water (1 1 v/v) After washing the column with acetone water (1 1 v/v) the product was eluted with acetone 4M acetic acid (1 1 v/v) until the effluent no longer reacted with Ehrlich s p dimethylaminobenzaldehyde reagent The effluent was concentrated to a syrup yielding the butoxycarbonylmethyl α ketoside of peracetylated *N* propionylneuraminic acid which crystallized on standing Recrystallization from hot acetone water (2 1 v/v) at room temperature yielded 200 mg of pure α ketoside chromatographically free from its β anomer

S_{dec} = 186–188 °C $[\alpha]_D^{25}$ = −15° (*c* – 1 0 in methanol)

Attempts to isolate a crystalline β anomer were unsuccessful

2 Aminoethylaminocarbonylmethyl α ketoside of N propionylneuraminic acid The butoxycarbonylmethyl α ketoside of peracetylated *N* propionylneuraminic acid (40 mg) was treated with anhydrous ethylene diamine (2 ml) at room temperature as described for preparation of the corresponding *N* acetylneuraminic acid derivative (5) The yield of 2 aminoethylaminocarbonylmethyl α ketoside of *N* propionylneuraminic acid was 20 mg

$[\alpha]_D^{25}$ = + 2° (*c* – 1 0 in water)

Column Experiments

Standard procedure for examination of different conjugates Columns of neuraminic acid derivative Sepharose conjugates (5 × 5 mm) were equilibrated with 0 1 M Tris maleate buffer pH 6 4 0 01 M as regards calcium chloride at 4° C Influenza virus vaccine (0 3 ml) was applied to the columns and was allowed to penetrate into the gels After about 15 min two 0 1 ml portions of buffer were added to each column and the viruses were eluted with the same buffer as above Fractions of 1 ml were collected at a flow rate of 0 2–0 3 ml/min A buffered solution of the benzyl α ketoside of *N* acetylneuraminic acid (0 4–2 mg) in water (50µl) was added to each fraction Neuraminidase activity was determined by incubating the fractions at 37° C for 30 60 min and 18 h with the 0 min value as a blank Samples (50–200 µl) were removed and released *N* acetylneuraminic acid was determined according to (8)

The viruses were desorbed from the columns by adding the benzyl α ketoside of *N* acetylneuraminic acid to the eluent (0 4–2 mg/ml) The effluent was incubated and analyzed for neuraminidase activity as above omitting the addition of substrate

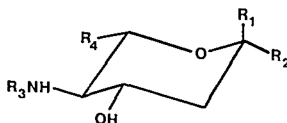
Enzyme activity remaining in the column was determined by incubation of the conjugate with substrate in a total volume of 1 ml as described above

Preparation of columns for affinity chromatography A column of the neuraminic acid derivative Sepharose conjugate (5 × 50 mm) was treated with crude formaldehyde inactivated influenza virus vaccine (3 ml) until saturation The column was washed with 0 1 M Tris maleate buffer pH 6 4 0 01 M as regards calcium chlor de at 4° C until no more neuraminidase activity was present in the effluent. The column was then washed with buffer (5 ml) containing the benzyl α ketoside of *N* acetylneuraminic acid (2 mg/ml) The column was again washed with the maleate buffer Affinity columns prepared in this way were used for the purification of crude influenza virus vaccine (0 1 ml) by reversible adsorption using buffer and substrate-containing eluent as described above Fractions (1 ml) of the effluent were collected and samples (50 μl) were analyzed for neuramin dase activity and for haemagglutination activity after dialysis against phosphate buffered saline

RESULTS AND DISCUSSION

A Sepharose bound nonulosaminic acid molecule represents a single neuraminidase binding site attached by a short chain to the matrix This conjugated gel should thus bind specifically to the neuraminidase or haemagglutinin of the influenza virus membrane In the present study small columns of different conjugates of neuraminic acid derivatives and Sepharose (Fig 1) were overloaded with formaldehyde inactivated virus purified by differential centrifugation in order to ensure maximal specific binding of virus to the sorbents The columns were washed with buffer to remove unbound virus Attempted desorption of ligand bound virus was performed by addition of benzyl α ketoside of *N*-acetylneuraminic acid to the buffer After completed elution the neuraminidase activity in the different effluent fractions and that remaining in the columns was determined

A column of *N* acetylneuraminic acid Sepharose type V (3) adsorbed influenza virus strongly Addition of large amounts of benzyl α ketoside of *N* acetylneuraminic acid (Fig 2 A) change of pH or elevation of temperature to 37° C, did not effectively induce desorption of the viruses In contrast it was found previously (3) that neurami nidase from *Vibrio cholerae* is eluted effectively by addition of substrate to the eluent The presence of intact virus in the *N* acetylneuraminic acid Sepha rose column was confirmed by electron microscopy Viruses were found only on the gel surface On incubation of the gel bound viruses with the benzyl α ketoside of *N* acetylneuraminic acid as sub strate a strong neuraminidase activity could be demonstrated However the enzyme activity was lower compared with that of unbound virus An inhibitory effect (60–70% inhibition) was also obtained by the addition of α *N* acetylneuraminic acid Sepharose to an incubation system containing virus and substrate



Derivative of neuraminic acid Sepharose conjugate	R_1	R_2	R_3	R_4
α N acetyl	COOH	OCH_2-Sepharose	CH_3CO	$CH_2OHCHOHCHOH$
7-C analogue of α N acetyl	COOH	OCH_2 Sepharose	CH_3CO	CH_2OH
β N acetyl	OCH_2-Sepharose	COOH	CH_3CO	$CH_2OHCHOHCHOH$
7-C analogue of β-N acetyl	OCH_2-Sepharose	COOH	CH_3CO	CH_2OH
α N propionyl	COOH	OCH_2 Sepharose	CH_3CH_2CO	$CH_2OHCHOHCHOH$
7-C analogue of α N propionyl	COOH	OCH_2 Sepharose	CH_3CH_2CO	CH_2OH

Fig 1 Different conjugates of neuraminic acid derivatives and Sepharose

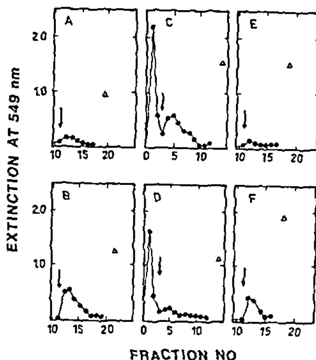


Fig 2 Activity of viral neuraminidase in the effluents from columns of neuraminic acid derivative Sepharose conjugates

Purified formaldehyde inactivated influenza virus vaccine (0 3 ml) was applied to columns of neuraminic acid derivative Sepharose(0 1 ml)

A = α *N* acetylneuraminic acid Sepharose
B = 7 C analogue of α *N* acetylneuraminic acid Sepharose
C = β *N* acetylneuraminic acid Sepharose
D = 7 C analogue of β *N* acetylneuraminic acid Sepharose
E = α *N* propionylneuraminic acid Sepharose
F = 7 C analogue of α *N* propionylneuraminic acid Sepharose

After being in contact with the gels for 15 *minutes* unbound viruses were washed away with 0 1 M Tris maleate buffer pH 6 4 0 01 M as regards calcium chloride at 4° C

Attempted desorption of the bound viruses was performed by the addition of 2 mg/ml of the benzyl α ketoside of *N* acetylneuraminic acid to the buffer The arrow denotes the addition of this neuraminidase substrate △ represents residual neuraminidase activity associated with the gel

Fractions of 1 ml were collected and samples of 50 μl were analyzed for neuraminidase activity Released *N* acetylneuraminic acid was determined according to (8) and expressed as extinction at 549 nm Incubation was performed for 18 h For details see Materials and Methods

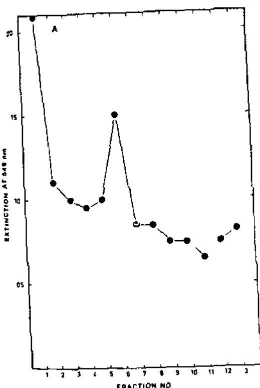
It has been reported previously that *Vibrio* to ian of *N* acetylneuraminic acid (3) Formaldehyde inactivated viruses were adsorbed readily to the latter conjugate at pH 6 4 Neuraminidase activity could be displaced from the column by the addition of benzyl α ketoside of *N* acetylneuraminic acid to the eluent (Fig 2 B) The lower affinity of this conjugate for the viruses as compared to that of the parent one was also reflected by a lower inhibitory effect on the enzyme activity (30–40% inhibition in an incubation system)

N acetylneuraminic acid β ketosides with neutral aglycones are resistant to viral and bacterial neuraminidases and do not inhibit the enzyme (1) However *Vibrio cholerae* neuraminidase is weakly adsorbed on the Sepharose bound β ketoside of *N* acetylneuraminic acid (5) Although the weak affinity of the β *N* acetylneuraminic acid Sepharose for neuraminidase may represent a specific interaction ion-exchange effects cannot be excluded When influenza virus was applied to a column of this conjugate a portion of the influenza viruses was bound strongly and could not be desorbed even with large volumes of substrate containing eluent or high concentrations of substrate The remainder was eluted from the column by retardation with the washing buffer However addition of benzyl α ketoside of *N* acetylneuraminic acid to the buffer during the washing procedure of the overloaded column resulted in a neuraminidase activity peak (Fig 2 C)

In order to diminish the affinity of the virus for the *N* acetylneuraminic acid conjugate the Sepharose bound β *N* acetylneuraminic acid was transformed to its 7-C analogue This conjugate however also adsorbed very strongly a large part of the viruses (Fig 2 D) A low inhibitory effect of Sepharose bound β *N* acetylneuraminic acid or its 7 C analogue on the viral enzyme activity could be detected (15–20% inhibition in an incubation system)

It has been demonstrated that α ketosides of *N* propionylneuraminic acid are cleaved much more slowly by bacterial and influenza virus neuraminidases than the corresponding ketosides of *N* acetylneuraminic acid (7) thus indicating a lower affinity of the neuraminidase for the *N* propionyl than for the *N* acetyl derivative The aminoethylaminocarbonylmethyl α ketoside of *N* propionylneuraminic acid was synthesized and coupled to Sepharose 4 B The matrix bound *N* propionylneuraminic acid was then converted into its 7 C analogue These conjugates also bound formaldehyde inactivated influenza virus strongly (Fig 2 E and F) The virus could partly be desorbed from the 7-C analogue by addition of the benzyl α ketoside of *N* acetylneuraminic acid to the eluent (Fig 2 F)

To demonstrate that neuraminidase eluted with



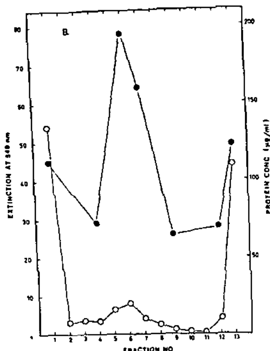


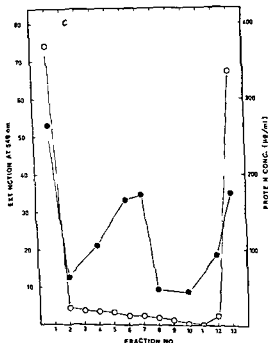
Fig 3 Sedimentation of different influenza virus preparations in sucrose density gradient

Fractions (1 ml) were collected and assayed for protein and neuraminidase activity *N* acetylneuraminic acid was determined after A 18 h B 5 h C 18 h of enzyme incubation For details see Material and Methods

A Influenza virus purified by affinity chromatography on 1 ml of the 7 C analogue of α *N* acetylneuraminic acid Sepharose according to the experiment described under Fig 4

enzyme substrate from the different columns is not a dissociation product from matrix bound viruses the material eluted with enzyme substrate was separated by centrifugation in a linear sucrose gradient Fig 3 shows that neuraminidase eluted from the matrix with enzyme substrate unpurified influenza virus vaccine in allantoic fluid and viruses purified by differential centrifugation all give a similar distribution of neuraminidase activity in the gradient The main activity is associated with intact influenza virus particles according to *Laver & Webster* (6)

The strong interaction between the influenza virus vaccine and the neuraminic acid derivative Sepharose conjugates is probably due to the



B Influenza virus purified commercially by differential centrifugation

C Crude preparation of influenza virus in allantoic fluid

● = neuraminidase activity

○ = protein concentration

presence of multiple neuraminidase molecules on the virus coat surface or is a result of the formaldehyde treatment of the virus preparation However, a portion of the ketosidically bound neuraminic acid in the conjugates seems to fulfil the steric requirements for a reversible adsorption of the viruses using neuraminidase substrate as desorbent

The binding properties of the conjugates were not influenced markedly by changing the pH-value of the buffer to 4 0 or 9 0 Saturation of the conjugates with benzyl α ketoside of *N* acetylneuraminic acid dissolved in buffer before addition of virus to the columns did not decrease the portion of virus which could not be desorbed by substrate

In order to prepare affinity columns suitable for the purification of virus vaccine a conjugate of the neuraminic acid derivative with Sepharose was thus first saturated with virus The column was washed with the buffer until the effluent was essentially free of neuraminidase activity then with buffer containing the benzyl α ketoside of *N* acetylneuraminic acid and finally with the buffer used initially After this treatment all sites which bind the viruses strongly are blocked and only those sites which bind the viruses reversibly are available Affinity columns prepared in this way were used repeatedly Fig 4 shows a typical experiment in which a 1 ml column of the 7-C analogue of α *N* acetylneuraminic acid Sepharose was used for separating formaldehyde inactivated influenza viruses from allantoic fluid components The recovery of the virus added to the column and eluted with 5 ml of substrate-containing buffer was more than 80% as determined by haemagglutination tests

In order to examine whether the formaldehyde inactivation of the viruses was responsible for the phenomenon that relatively large amounts of viruses could not be desorbed from the columns by neuraminidase substrate an experiment with freshly prepared non inactivated viruses was performed A column of α *N* acetylneuraminic acid Sepharose was saturated with non inactivated virus and after washing with buffer the viruses were

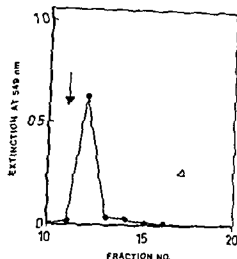


Fig 5 Affinity chromatography of non inactivated influenza virus

Crude non inactivated influenza virus vaccine (1 ml) was applied to a column of α *N* acetylneuraminic acid Sepharose (0 1 ml) The column was washed with 10 ml of buffer Viruses were then desorbed with 5 ml buffer containing 2 mg/ml benzyl α ketoside of *N* acetylneuraminic acid

The arrow denotes the start of the addition of substrate Fractions of 1 ml were collected and samples of 50 µl were analyzed for neuraminidase activity Released *N* acetylneuraminic acid was determined according to (8) and expressed as extinction at 549 nm △ represents residual neuraminidase activity associated with the gel Incubation was performed at 37° C for 4 h in the presence of benzyl α ketoside of *N* acetylneuraminic acid

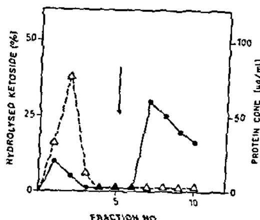


Fig 4 Purification by affinity chromatography of influenza virus cultured in allantoic fluid

A column of the 7 C analogue of α *N* acetylneuraminic acid Sepharose gel (1 ml) was saturated with formaldehyde inactivated influenza virus and then washed consecutively with buffer and substrate containing buffer as described under Materials and Methods Crude influenza virus vaccine (0 1 ml) containing allantoic fluid components was applied to this column The column was washed with 5 ml of the buffer Viruses were then desorbed with 5 ml of buffer containing 2 mg/ml benzyl α ketoside of *N* acetylneuraminic acid

The arrow denotes the start of the addition of substrate Fractions of 1 ml were collected and samples of 50 µl were analyzed for neuraminidase activity (●) according to (8) Incubation was performed at 37° C for 18 h in the presence of benzyl α ketoside of *N* acetylneuraminic acid The protein content (△) of the effluent fractions was determined by a modified Lowry method according to (2) using human serum albumin as standard

eluted with substrate-containing buffer as described under Fig 2 As shown in Fig 5 a more complete desorption was obtained with non inactivated viruses than in the corresponding experiment with formaldehyde treated viruses (Fig 2 A) Considering the inhibitory effect of the α N acetylneuraminic acid Sepharose on the neuraminidase a recovery of 40 per cent of non inactivated virus was obtained from this gel

The present procedures for obtaining pure influenza viruses from crude preparations may also be used in physicochemical and immunological studies of the viruses and further work in this field is in progress

We thank Mrs *Ingegerd Friberg* (National Defence Research Institute) and Mrs *Maria Hilme* (King Gustaf V Research Institute) for skilful technical assistance

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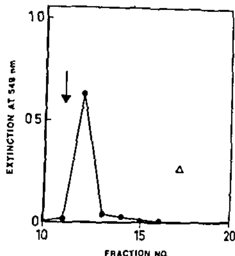


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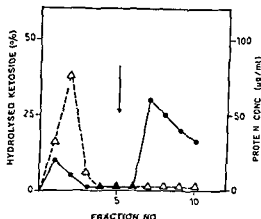


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Acta path microbiol scand Sect B 87 137–140 1979

STUDIES ON *YERSINIA ENTEROCOLITICA* ISOLATED FROM SWINE AND DOGS

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Pedersen K B & Winblad S Studies on *Yersinia enterocolitica* isolated from swine and dogs Acta path microbiol scand Sect B 87 137–140 1979

Yersinia enterocolitica serotype O3 was isolated from the colon contents of 27 (4 5%) of 599 bacon pigs on slaughter Two (1 7%) out of 115 dogs were positive by culture for this human pathogenic serotype of *Y enterocolitica* A number of other serotypes of Y *enterocolitica* were also obtained both from porcine and canine enteric contents (4b 5a 6 7 11 12 15 17 19 26b and non typeable strains) All these serotypes are supposedly non pathogenic for man and may possibly originate from the microflora of the environment Haemolysis on bovine blood agar was observed for strains belonging to O serotype 3

Key words Yersinia enterocolitica swine dogs

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Yersinia enterocolitica is apparently widely distributed in the digestive tract of man and animals and also in the environment A certain relationship seems to exist between serological pattern biotype and occurrence in certain hosts Thus serotypes O3 O8 and O9 are well established as human pathogens while serotype O1 is the most prevalent in chinchillas and O2 in hares and goats However the reservoirs and the means of spreading of *Y enterocolitica* are largely unknown

The aim of the present work was to study the occurrence of *Y enterocolitica* in swine and dogs and to characterize the strains biochemically and serologically in order to elucidate further the fragmentarily known epidemiology of these bacteria Special attention was paid to serotype O3 which is the predominant human pathogenic type in Denmark (1 3)

MATERIALS AND METHODS

Material I The colon contents of 399 bacon pigs slaughtered during 1976 at one abattoir were examined for *Y enterocolitica*

Material II The colon contents of 200 bacon pigs slaughtered at the same time and at the same abattoir as those of material I were examined only for *Y enterocolitica* serotype O3 the common human pathogenic type

Material III A total of 215 faeces samples and 171 rectal swabs from dogs submitted for veterinary examination in Copenhagen were examined for *Y enterocolitica* No selection was made for special clinical diagnoses Of the faeces samples 115 were examined both by culture and by the fluorescent antibody technique while the remaining 100 and the 171 rectal swabs were examined by the fluorescent antibody technique and cultured only if found positive by that method

Enrichment and culture 1 g colon contents or faeces was suspended in 10 ml phosphate buffered saline (pH 7 6) and stored at 4° C for 3 weeks Rectal swabs were immersed in 2 ml PBS and stored as above From each of these cold-enrichment cultures two surface cultures were made on MacConkey agar One was incubated at 22° C for 48 hours the other at 37° C for 24 hours and at 22° C [illegible]

[illegible] as being *Y enterocolitica* suspect Gram negative rods motile at 22° C but not at 37° C urease positive phenylalanine negative ornithine-decarboxylase positive nitrate-positive and citrate negative were identified

TABLE 2 *Biochemical[a]) and Serological Characteri ation of* Y enterocolitica *from Sw ine*

Serotype	3	11 12	5a 6 7 15 19 Non typeable	26b	4b	17	Non typeable
Indole	−	+w	+	+	+	+	
Aesculin	−	−	+	+	+	+	+
Salicin	−	−	+	+	+	+	+
Voges Pr 37° C	−	−	−	−	−	−	−
Voges Pr 22° C	+	−	+	+	+	+	−
Rhamnose	−	−	−	(+)	+	+	(+)
Sucrose	+	−	+	+	+	+	−
Lactose	−	−	−	(+)	(+)	(+)	−
d Xylose	−	+	+	+	+	+	+
Trehalose	+	+	+	+	+	+	+
Cellobiose	+	+	+	+	+	+	+
Melibiose	−	−	−	(+)	(+)	−	−
Lecithinase	−	or +w	+	+	+	+	−
Citrate	−	−	−	−	−	−	−
Biotype	4	[illegible]	1	1	1	1	1
No of strains	27	4	41	1	3	1	1

a) The biochemical tests were performed at 37° C except for Voges Proskauer which was also carried out at 22° C Lecithinase was carried out at 22° C
\+ positive within 3 days
(+) delayed positive
negative after incubation for 21 days
w weak reaction

Biochemical observations As shown in Table 2 the four strains belonging to O serotypes 11 and 12 did not ferment sucrose or produce acetoin The inability of strains of these serotypes to ferment sucrose is well known and seems to be a special characteristic The rhamnose positive strains all utilized citrate at 22° C but not at 37° C Variations dependent on temperature were observed in fermentation of raffinose rhamnose and melibiose Some of the rhamnose positive strains showed delayed fermentation of lactose

An additional observation was that all the strains belonging to O serotype 3 showed distinct beta haemolysis on bovine blood agar after storage of the cultures at room temperature for 3–12 days

DISCUSSION

The human pathogenic O3 serotype of *Y enterocolitica* was isolated from the colon contents of 27 (4 5 per cent) of 599 bacon pigs on slaughter There was no evidence to suggest that this organism was pathogenic for the swine themselves The frequency observed is in agreement with a preliminary report (4) and is comparable with frequencies reported from countries such as *Belgium* (9) Canada (6) and Japan (12)

Other studies by *Pedersen* (5) have shown that 30 per cent of Danish pigs carry *Y enterocolitica* serotype O3 in their throats In Belgium *Wauters & Janssens* (8) isolated O3 from 53 per cent of pig tongues bought in butchers shops All these observations suggest that pigs may be one of the sources of *Y enterocolitica* infection in man In the present study serotype O3 was isolated from only two of the 115 dogs examined

Hitherto serotype O9 has not been isolated from the enteric contents or throats of Danish swine As ment oned ...

with this

... pathogenic for man (4b 5a, 6 7 11 12 15 17 19 26b and non typeable strains) were cultivated in 51 cases Most of these isolates belonged to O serotypes 5a and 6

as *Y enterocolitica* and subjected to further biochemical characterization and serological typing

Biochemical reactions Biochemical tests were perfor med at 37° C unless otherwise stated Fermentation of carbohydrates was performed in meat extract peptone broth with 1 per cent of the test substances in question Bromthymol blue was used as indicator Negative tests were retained for 21 days before being discarded

Fluorescent antibody technique Three week old cold enrichment cultures were examined for *Y enterocolitica* serotype O3 by the indirect fluorescent antibody technique

Serological typing After biochemical testing suspec ted *Y enterocolitica* strains were serotyped by slide agglutination tests with specific absorbed rabbit O antisera for O serotypes 1–26 (7 10 11)

Phagetyping was performed by Prof *H H Mollaret* Institut Pasteur Paris

RESULTS

Material I A total of 68 strains of *Y enterocolitica* were isolated from 399 samples of colon contents collected from bacon pigs on slaughter during January April July and October (Table 1) Seventeen strains were O serotype 3 the common human pathogenic type Five strains were non typeable Except for 4 strains falling into O serotypes 11 and 12 the rest belonged to the in dole aesculin and salicin positive types of *Y enterocolitica* Of these 13 strains were referred to O serotype 6 and 12 to O serotype 5a Serotypes O4b O7 O15 O17 O19 and O26b were also represent ed Seasonal variation was not observed

Material II Ten strains of O serotype 3 were isolated from the colon contents of 200 pigs viz 2 out of 100 in September 3 out of 50 in November and 5 out of 50 in December

On pooling the above results it can be seen that 27 strains of the human pathogenic O3 serotype of *Y enterocolitica* were isolated from 599 pigs (4 5 per cent) From the 399 samples in Material I 51 strains of other serotypes of *Y enterocolitica* (12 8 per cent) were cultured Presumably these bacteria are indigenous to the normal flora of pigs and are not pathogenic for human beings Three porcine *Y enterocolitica* strains of serotype 3 were phagetyped and all found to belong to phagetype VIII

Comparison between culture and IFA test for demonstration of Y enterocolitica *serotype 3* A total of 499 specimens were examined by both methods Nineteen samples were positive by IFA Among the 480 specimens negative by IFA 5 were positive by culture Of the samples positive by IFA 18 could be verified by culture 9 in the primary culture 8 after repeated culture and one after repeated culture in enrichment medium at 4° C for three weeks One could not be verified

Material III Of the 215 faeces samples from dogs 115 were examined both by IFA test (for diagnosis of *Y enterocolitica* serotype O3) and by culture (for diagnosis of all serotypes of *Y enterocolitica*) *Y enterocolitica* was isolated in 7 cases (6 1%) Three strains belonged to serotype 5a (biotype 1) and 2 to serotype 6 (biotype 1) Two strains were identified as the human pathogenic O serotype 3 The specimens from which the two last mentioned strains originated were negative by IFA test The two dogs carrying O serotype 3 were 4½ months and 3 years old respectively The ages of the animals from which the other probably non pathogenic strains were isolated varied between 4½ and 8 years

In addition examination was made of 100 faeces samples and 171 rectal swabs from dogs initially by the IFA test only with special regard to O serotype 3 In 9 cases the IFA test was positive and in 11 cases doubtful Only one of these 20 cases was positive by culture This was a sample with doubtful result in the IFA test and culture was positive only after repeated enrichment

TABLE 1 *Isolation of* Y enterocolitica *from Colon Contents of Swine*

	No examined	No of *Y enterocolitica* positive samples (%)	O serotype 3	4b	5a	6	7	11	12	15	17	19	26b	Non typeable
January	100	17 (17)	4		3	4	1		2		1	1	1	
April	99	22 (22)	2		4	4	3	1	1			2		5
July	100	19 (19)	6	3	3	2	3			1		1		
October	100	10 (10)	5		2	3								
Total	399	68 (17)	17	3	12	13	7	1	3	1	1	4	1	5

STUDIES ON THE INTERACTION BETWEEN DIFFERENT O-SEROTYPES OF *YERSINIA ENTEROCOLITICA* AND HeLa CELLS

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Pedersen K B Winblad S & Bitsch V Studies on the interaction between different O serotypes of *Yersinia enterocolitica* and HeLa cells Acta path microbiol scand Sect B 87 141–145 1979

Eighty strains of *Yersinia enterocolitica* were examined for their ability to invade HeLa cells The following O serotypes were found to be invasive 1 3 4a 4c 5b 8 9 15a 18 20 21 and 22 Biochemically these types are either indole aesculin and salicin negative or indole positive but aesculin and salicin negative Other serotypes of Y enterocolitica did not invade HeLa cells Biochemically these non invasive strains are indole aesculin and salicin positive Also the non sucrose fermenting strains of O serotypes 11 and 12 were non invasive The interaction with HeLa cells was not restricted to viable bacteria since also bacterial cells inactivated by formalin or ultraviolet light could gain entrance to the HeLa cells Heating of the bacteria to 100° C for 30 min completely inhibited their invasiveness Monolayer cultures of porcine kidney cells were also invaded by strains which invaded HeLa cells

Key words *Yersinia enterocolitica* HeLa cells

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The ability of certain strains of *Yersinia enterocolitica* to invade HeLa cells has been reported by *Lee et al* (2) *Une* (4) and *Une et al* (5) Since only strains otherwise recognised as pathogenic were found to be invasive to HeLa cells this feature would appear to be useful in evaluation of the pathogenicity of strains of *Y enterocolitica* It might also be of some significance for differentiation of the heterogeneous species of *Y enterocolitica*

In this work O serotypes 1–26 of *Y enterocolitica* were tested for interaction with HeLa cells In addition some of the strains were examined for interaction with cultures of porcine kidney cells The role of the viability of the bacteria for interaction was examined through inactivation of the bacteria in different ways

MATERIALS AND METHODS

Bacteria Eighty strains of different *O-serotypes* and biotypes of *Y enterocolitica* from the culture collection of the Institute of Clinical Bacteriology at Malmo were used The identity of the strains can be seen from Table 1 The O-serotypes were identified according to *Winblad* (7 8) and *Wauters et al* (6) the biotypes according to *Nilehn* (3)

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[illegible] and washed three times with that solution Finally they were resuspended in saline to a

mically they are indole-, aesculin- and salicin-positive, except for the four strains belonging to O-serotypes 11 and 12, which, unlike all other serotypes, do not ferment sucrose

The present findings in dogs are consistent with the results obtained by *Kaneko et al* (2), who isolated *Y enterocolitica* from 25 (5 5 per cent) of 451 dogs examined In the Japanese study, in which four regions of the intestinal tract were examined from each dog, it was remarkable that when present, serotypes O3, O5b and O9 were usually isolated from more than three regions per dog In contrast, serotypes O5a and O6 were never isolated from more than one region thus indicating that probably these types occur only transitorily in the intestinal flora

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The authors are indebted to Mrs *Sonja Jensen* Small Animal Clinic Royal Veterinary and Agricultural University and to Dr *H V Nørrung* State Veterinary Serum Laboratory for supplying dog faeces samples Thanks are also due to Professor *H H Mollaret* Institut Pasteur Paris for phagetyping We acknowledge the technical skill of Mrs *Minna Petersen*

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STUDIES ON THE INTERACTION BETWEEN DIFFERENT O-SEROTYPES OF *YERSINIA ENTEROCOLITICA* AND HeLa CELLS

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Pedersen K B Winblad S & Bitsch V Studies on the interaction between different O serotypes of *Yersinia enterocolitica* and HeLa cells Acta path microbiol scand Sect B *87* 141-145 1979

Eighty strains of *Yersinia enterocolitica* were examined for their ability to invade HeLa cells The following O serotypes were found to be invasive 1 3 4a 4c 5b 8 9 15a 18 20 21 and 22 Biochemically these types are either indole aesculin and salicin negative or indole positive but aesculin and salicin negative Other serotypes of *Y enterocolitica* did not invade HeLa cells Biochemically these non invasive strains are indole aesculin and salicin positive Also the non sucrose fermenting strains of O serotypes 11 and 12 were non invasive The interaction with HeLa cells was not restricted to viable bacteria since also bacterial cells inactivated by formalin or ultraviolet light could gain entrance to the HeLa cells Heating of the bacteria to 100° C for 30 min completely inhibited their invasiveness Monolayer cultures of porcine kidney cells were also invaded by strains which invaded HeLa cells

Key words *Yersinia enterocolitica* HeLa cells

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strains otherwise recognised as pathogenic were found to be invasive to HeLa cells this feature would appear to be useful in evaluation of the pa h

... were tested for interaction with HeLa cells In addition some of the strains were examined for interaction with cultures of porcine kidney cells The role of the viability of the bacteria for interaction was examined through inactivation of the bacteria in different ways

MATERIALS AND METHODS

Bacteria Eighty strains of different O-serotypes and biotypes of *Y enterocolitica* from the culture collection of the Institute of Clinical Bacteriology at Malmo were used The identity of the strains can be seen from Table *1* The O serotypes were identified according to *Winblad* (7 8) and *Wauters et al* (6) the biotypes according to *Nilehn* (3)

TABLE 1 *Strains of* Yersinia enterocolitica *Tested for Interaction with HeLa Cells*

O serotype	Biotype	Strain identity	Source	Interaction[a] with HeLa cells
1	3	P 348 Py 204 Becht 51/6	Chinchilla	+
1	3	Py 1422 Schneider	Hare	+
2	5	Py 10 P 301 Lucas	Hare	–
2	5	Py 11 P 303 Lucas	Hare	–
2	5	Py 420 Weaver	Hare	–
2	5	P 368 Py 14 Lucas 404	Hare	–
2	5	Ny 35 Lassen	Goat	?
2	5	Ny 39 Lassen	Goat	–
2a	1	Ny 21 Lassen	Water	–
2a	1	Ny 22 Lassen	Water	–
2a	1	Ny 23 Lassen	Water	–
3	4	My 0 »Winblad« Winblad	Homo	+
3	4	96 13/76 Pedersen	Swine	+
3	4	2429 4/76 Pedersen	Swine	+
3	4	3629 12/76 Pedersen	Swine	+
3	4	1188/75 Pedersen	Dog	+
3	4	759/76 Pedersen	Dog	+
4a	3	P 76 Knox 1960	Chinchilla	+
4b	1	Fy 52 Ahvonen 1028/71	Homo	–
4b	1	Ny 26 Lassen 619 D	Water	–
4b	1	2477 23/76 Pedersen	Swine	–
4b	1	2828 16/76 Pedersen	Swine	–
4c	2	Ca 4 Cockroft	Homo	+
4c	2	Ca 5 Cockroft	Homo	+
5a	1	Py 123 Institut Pasteur	Cow	–
5a	1	P 1402 Institut Pasteur	Milk	–
5a	1	144 30/76 Pedersen	Swine	–
5a	1	1758 3/76 Pedersen	Swine	–
5a	1	873 194/77 Pedersen	Dog	–
5b	3[b]	P 77 Knox 1048	Chinchilla	+
6	1	P 219 Py 102 Bojsen Moller	Homo	–
6	1	96 14/76 Pedersen	Swine	–
6	1	1758 5/76 Pedersen	Swine	–
6	1	873 190/77 Pedersen	Dog	–
7	1	P 413 Py 107 Borg Petersen	Guinea pig	–
7	1	My 9 Nilehn	Homo	–
7	1	Chy 80 Aldova 17395	Microtus	–
7	1	Day 69 Pedersen 2477 19/76	Swine	–
7/8	1	Day 3 Pedersen 1106/75	Dog	–
7/13	1	Day 74 Pedersen 2828 29/76	Swine	–
8	2	P 311 Schleifstein Albany 5819	Homo	+
9	3	My 79 Nilehn 5385	Homo	+
10	1	Py 474 Wauters 4/69	Homo	–
11	?	P 226 Kristensen	Homo	–
11	?	Py 1420 Schneider	Fox	–
11	?	USA 10 Weaver B 3013	Homo	–
11	?	Day 60 Pedersen 1758 27/76	Swine	–
12	?	Py 490 Lucas 63	Hare	–
12	?	Ny 27 Lassen	Water	–
12	?	Ca 17 Toma 149	Homo	–
12	?	96 12/76 Pedersen	Swine	–
13	1	Py 553 Wauters	Homo	–
14	1	Py 480 Graux	Homo	–
15	1	USA 19 Py 849 Weaver	Homo	–
15	1	Py 1663 Hausnerova 8811	Homo	–
15	1	Day 61 Pedersen 2429 12/76	Swine	–

15	1	Sva 25 Thal	Swine	−
15	1	Day 134 K K Kristensen	Water	−
15a	4	Py 614 Esseveld 188	Homo	+
16	1	P7 867 Graux	Homo	−
17	1	Ny 20 Lassen 333 B	Water	−
17	1	USA 40 Weaver C 3741a	Homo	−
17	1	USA 46 Malovany 48	Homo	−
17	1	USA 48 Malovany 50	Homo	−
17	1	Ca 8 Toma	Water	−
17	1	144 22/76 Pedersen	Swine	−
18	2	USA 16 Weaver B 4542	Homo	+
19	1	Fy 50 Ahvonen 534	Homo	−
19	1	144 4/76 Pedersen	Swine	−
19	1	1758 2/76 Pedersen	Swine	−
20	2	USA 15 Weaver B 4403	Homo	+
21	2	Ca 18 Toma	Homo	+
22	2	USA 30 Weaver C 109	Homo	+
23	1	Py 1636 Hausner 3308	Water	−
24	1	Py 1655 Hausner 3833	Water	−
25	1	Py 1668 Hausner 8931	Water	−
26	1	Py 1662 Hausner 8459	Water	−
26	1	Py 1638 Hausner 3310	Water	−
26	1	Py 1667 Hausner 8930	Milk	−
26	1	Py 1671 Hausner 8952	Water	−
26	1	144 32/76 Pedersen	Swine	−

a) + Interaction with HeLa cells
− No interaction with HeLa cells

b) Previously referred to Nilehn s biotype 2 (3)

density of approximately 8 × 10^8 bacteria per ml One portion was heated to 100° C for 30 minutes another was irradiated with ultraviolet light for 15 minutes and a third was inactivated with 0 3% formalin washed and then resuspended in sterile saline One portion was left untreated for control All the inactivated suspensions were tested for sterility Suspensions which had been heated at 100° C for 30 min were considered to be devoid of H antigen while after other forms of inactivation the H antigen was assumed to have been preserved

HeLa cells Monolayers of HeLa cells were grown in Leighton tubes with coverslips Medium 199 (Earle s salts) with HEPES buffer sodium b carbonate and 10 per cent calf serum was used The cells were grown at 37° C without use of antibiotics

Porcine kidney cells The established cell line PK 15 and secondary pig kidney cells were used The growth conditions were as for HeLa cells

Infection of cell cultures The experiments were carried o it n Le ghton tubes w th coversl ps The [illegible] antibiotics was added After incubation at 37° C for 90 minutes the inoculum was removed and the cell cultures were washed three times with warm PBS to remove extracellular bacteria The coversl ps were transferred to new Leighton tubes and incubated with 2 ml fresh Medium 199 with 10 per cent calf serum and kanamycin (50 µg/ml) for 3 hours at 37° C After this incubation period the medium was removed and the cell cultures washed three times with PBS (4° C) and then fixed with methanol for 1 min With a few of the non invasive strains many bacteria adhered extracellularly but this phenomenon was usually absent when washed suspen sions of bacteria were used as inocula instead of nutrient broth cultures The preparations were stained with Giemsa solution mounted on slides and examined by oil immersion microscopy at a magnification of 630 ×

RESULTS

Clear distiction could be made between the strains of *Y enterocolitica* which were able to interact with HeLa cells and those which were not With interacting strains a very great number of bacteria were present apparently intracellularly in the majority of cells in the monolayer cultures However strain-dependent variations in the invasi veness were observed

TABLE 1 *Strains of* Yersinia enterocolitica *Tested for Interaction with HeLa Cells*

O serotype	Biotype	Strain identity	Source	Interaction[a)] with HeLa cells
1	3	P 348 Py 204 Becht 51/6	Chinchilla	+
1	3	Py 1422 Schneider	Hare	+
2	5	Py 10 P 301 Lucas	Hare	−
2	5	Py 11 P 303 Lucas	Hare	−
2	5	Py 420 Weaver	Hare	−
2	5	P 368 Py 14 Lucas 404	Hare	−
2	5	Ny 35 Lassen	Goat	?
2	5	Ny 39 Lassen	Goat	
2a	1	Ny 21 Lassen	Water	−
2a	1	Ny 22 Lassen	Water	−
2a	1	Ny 23 Lassen	Water	−
3	4	My 0 › Winblad Winblad	Homo	+
3	4	96 13/76 Pedersen	Swine	+
3	4	2429 4/76 Pedersen	Swine	+
3	4	3629 12/76 Pedersen	Swine	+
3	4	1188/75 Pedersen	Dog	+
3	4	759/76 Pedersen	Dog	+
4a	3	P 76 Knox 1960	Chinchilla	+
4b	1	Fy 52 Ahvonen 1028/71	Homo	−
4b	1	Ny 26 Lassen 619 D	Water	−
4b	1	2477 23/76 Pedersen	Swine	−
4b	1	2828 16/76 Pedersen	Swine	−
4c	2	Ca 4 Cockroft	Homo	+
4c	2	Ca 5 Cockroft	Homo	+
5a	1	Py 123 Institut Pasteur	Cow	−
5a	1	P 1402 Institut Pasteur	Milk	−
5a	1	144 30/76 Pedersen	Swine	−
5a	1	1758 3/76 Pedersen	Swine	−
5a	1	873 194/77 Pedersen	Dog	−
5b	3[b)]	P 77 Knox 1048	Chinchilla	+
6	1	P 219 Py 102 Bojsen Møller	Homo	−
6	1	96 14/76 Pedersen	Swine	−
6	1	1758 5/76 Pedersen	Swine	−
6	1	873 190/77 Pedersen	Dog	−
7	1	P 413 Py 107 Borg Petersen	Guinea pig	−
7	1	My 9 Nilehn	Homo	−
7	1	Chy 80 Aldova 17395	Microtus	−
7	1	Day 69 Pedersen 2477 19/76	Swine	−
7/8	1	Day 3 Pedersen 1106/75	Dog	−
7/13	1	Day 74 Pedersen 2828 29/76	Swine	−
8	2	P 311 Schleifstein Albany 5819	Homo	+
9	3	My 79 Nilehn 5385	Homo	+
10	1	Py 474 Wauters 4/69	Homo	−
11	?	P 226 Kristensen	Homo	−
11	?	Py 1420 Schneider	Fox	−
11	?	USA 10 Weaver B 3013	Homo	−
11	?	Day 60 Pedersen 1758 27/76	Swine	−
12	?	Py 490 Lucas 63	Hare	−
12	?	Ny 27 Lassen	Water	−
12	?	Ca 17 Toma 149	Homo	−
12	?	96 12/76 Pedersen	Swine	−
13	1	Py 553 Wauters	Homo	−
14	1	Py 480 Graux	Homo	−
15	1	USA 19 Py 849 Weaver	Homo	−
15	1	Py 1663 Hausnerova 8811	Homo	−
15	1	Day 61 Pedersen 2429 12/76	Swine	−

negative or indole positive but aesculin and salicin negative The latter fermentation pattern is common for strains isolated in the U S A or Canada

As regards O-serotypes 3 5b 8 and 9 the present findings confirm the work performed by *Une* (4) and *Une et al* (5) Using electron microscopy *Lee et al* (2) demonstrated that the interaction consists in penetration into the cells and not only in adherence to the HeLa cell membranes

The ability to interact with tissue culture cells is not restricted to viable bacterial cells since organisms inactivated by formalin or ultraviolet light were still capable of gaining entrance into HeLa cells These results might suggest that treatment which leaves the H antigen intact will not destroy the ability to interact with HeLa cells *Lee et al* (2) found that the invasiveness of some strains was most pronounced when the bacteria were grown at 22° C and almost nil when they were grown at 36° C [illegible]

since also inactivated bacteria were found to gain entrance to the HeLa cells The infectivity of the organisms for monolayer cell cultures was not limited to cells of human origin since also porcine kidney cells were invaded

As stated by *Lee et al* (2) the HeLa cell test is simple inexpensive and rapid and would seem to be useful in examination of isolates from clinical cases for possible pathogenic significance This may involve a further extension of the use of cell cultures in evaluation of bacterial pathogenicity

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O serotypes of *Y enterocolitica* that are usually considered pathogenic for man or animals were found to interact with the HeLa cells (Table 2) This was the case with O serotypes 1, 3, 4a 4c, 5b, 8, 9, 15a, 18, 20, 21 and 22 The strains belonging to O-serotypes 4c, 8, 18, 20, 21 and 22 were isolated from man in the U S A or Canada With the porcine strains of serotype O3, which are relatively freshly isolated, damage to and detachment of the HeLa cells was observed However, this cytotoxic effect was not observed if the process was terminated after the initial incubation for 90 minutes These O3 strains from pigs also showed a tendency to adhere to the glass and cell surfaces

The remaining O serotypes were negative in the HeLa cell test, including the non sucrose-fermenting strains of O serotypes 11 and 12, and all the indole positive, aesculin- and salicin fermenting, lecithinase positive strains It is of special interest that the strains of O serotype 17 were negative in the HeLa cell test In preparations with the Norwegian strain Ny 35, so many extracellularly adhering bacteria were present that the question of invasiveness was left open

The experiments with inactivated bacteria showed that cells exposed to 100° C for 30 min lost their ability to interact with the HeLa cells Inactivation with ultraviolet light or formalin did not disturb the interaction with HeLa cells The invasiveness of some of the strains (O1/P348 O3/My 0, O8/P311, O9 My 79) was examined also with bacteria grown at 37° C. In all cases a weaker interaction was found than with bacteria grown at 22° C

Four strains (O1/P348, O3/My 0 O3/96-13 O3/1188) which were positive, and one (O7/Dav 69) which was negative in the HeLa cell test, were examined for interaction with porcine kidney cells. The HeLa-cell invading strains also invaded porcine kidney cells while this was not the case with the strains which did not invade the HeLa cells No differences in this respect were observed between the porcine kidney cell line and secondary porcine kidney cells

DISCUSSION

Strains of *Y enterocolitica* which are generally considered to be pathogenic for man or animals were found to interact with HeLa cells (O serotypes 1, 3, 8 and 9) but also serotypes with a more uncertain pathogenicity were positive (O serotypes 4a, 4c, 5b, 15a, 18, 20, 21 and 22) The positive strains all belong to the biotypes of *Y enterocolitica* which are either indole-, aesculin and salicin

TABLE 2 *Interaction Between Different O serotypes and Biotypes of* Yersinia enterocolitica *and HeLa Cells*

Invasiveness of Y e Strains	O serotype	Biotype		Comments
Positive	1 3 4a 5b 9 15a	3 4	− − − ± + − − +	Human pathogenic strains and strains isolated from chinchillas and hare
	8 4c 18 20 21 22	2	+ − − + + + − +	Strains isolated from man in U S A or Canada
Negative	11 12	?	− − − + + − − −	Non sucrose fermenting strains
	2a 4b 5a 6 7 7/8,7/13 10 13 14 15 16 17 19 23 24 25 26	1	+ + + + + + ± +	Strains mostly isolated from animals and water Supposed to be non pathogenic. Also strains of serotype O17 isolated from a variety of human disease conditions (1)
	2	5	− − − − − − − +	Strains isolated from hares at goats Trehalose-negative

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BIOLOGICAL INDICATORS FOR THE CONTROL OF ETHYLENE OXIDE STERILIZATION

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Christensen E A & Kristensen H Biological indicators for the control of ethylene oxide sterilization Acta path microbiol scand Sect. B 87 147–154 1979

A new biological indicator has been developed for the control of ethylene oxide sterilization particularly for large scale sterilization of disposable medical equipment The aim has been to provide the new indicator with the same resistance to the combined effect of ethylene oxide and water vapour as the biological indicator referred to by the health authorities in Scandinavia The reference indicator contains spores of a Danish test strain *Bacillus subtilis* in sand The new one contains spores of a test strain used extensively for biological ind cators viz *B subtilis var niger (B globigii)* The spores in the new preparation are dried in pieces of cotton yarn The two indicators were exposed to ethylene oxide and water vapour in five different series of experiments and almost the same resistance was found In simulated routine sterilization procedures the new indicator was placed at locations not easily accessible for the gas and water vapour and the results reflected the blockage of diffusion The experiments included samples of household dust The resistance of the microorganisms in the dust was compared with that of the biological indicators Based on these comparisons it is concluded that the resistance of the two biological indicators to ethylene oxide is in accordance with the official Scandinavian standard for sterilized medical equipment when used in the control of sterilization of products with low microbial contamination

Key words Ethylene oxide sterilization control biological indicators

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method is used for industrial sterilization of disposable medical devices and supplies In the United States of America and in some of the West European countries the method is also used extensively at hospitals for sterilization of utensils However at hospitals in the Nordic countries ethylene oxide sterilization has never been accepted as a routine method

The inactivating effect of ethylene oxide on microorganisms of all kinds is well-documented (6 7 13) The microbiological effect is dependent on the concentration of ethylene oxide the exposure time the temperature and the relative humidity These parameters can be regulated to some degree during the sterilization procedures but the concen trations of ethylene oxide and water in the products to be sterilized become adjusted relatively slowly to the concentrations that can be measured in the chamber of the sterilizer A state of balance within the sterilization period is not always achieved and physico-chemical determination of the decisive parameters in the ethylene oxide sterilization process is an unreliable measure of the microb olo

corresponding to that of the biological indicator (IS) obtainable from Statens Institutt for Folkehelse in Oslo Statens Bakteriologiska Laboratorium in Stockholm and Statens Seruminstitut in Copenha gen

TABLE 1 *E h lene Ox de Trea men n S e l er A The F gures Shown a e he Sum of he Resul s of Ind dual Expe men s w h h Gene ally Included 6 o 12 of each of he Th ee Bac e iolog al Prepa a ons*

Type o ind ca or		E posu e time: 20 m nu es — Tota number of samp es	Number of samp es w h grow h	Numbe of rv ng colony f rm ng un ts per sample	2–4 hours — Total numbe of samp es	N mbe of samp es w th growth	N mbe of s rv ng co ony forming un ts pe samp e	8 22 hours — Tota number of samp es	Number of samp es w h g ow h	Number o urv ng colony form ng un ts pe sample
hou pre m d ca n	D samp es	54	36		8	02		25	12	
	IS	2 6	2 6	0	342	342	10	215	2 5	0
	IF	2 0	2 0	0 0	234	28	0 0	252	25	10 0
re humidi cation a 6°C 4 hours	Du samp es	68	143		167	2		156	2	
	S	240	6		246	109		234	106	
	F	284	05		280	32		240	2	

Ethy ene ox de concen a on 450 mg pe l tre rela ve hum d y 60% tempe a u e 54 C The chamber conta ned only the bacte o og cal p eparations

S er e B w h a o ume of 80 l es

Hea ng of he p odu o be e zed take p ace by means of sa u a ed wa e apou *adm ed o he* chambe du ng a edu on n empe a u e f om app ox 95 C o 54 C ± 1 C The empera u e n he chambe s kep constan a 54 C ± 1 C by a wa er jacket a ound he chambe The p ocess started by e acua ng he a o a esidual p essure of 5 mm Hg (20–40 mm H on y f he e s a la ge quan y of p per o ex es n he chambe) Wa e vapou hen n oduced *o abou* 00% e a e hum d y The e hy ene ox de f eon m x u e adm ed mmed a ely af e wa d as n A The mes of exposu e and he subsequen e acua on a e as desc bed n A

RESULTS

In the *f rs ser es of exper men s* wh ch were a cont nuat on of the p lot study carr ed out to es abl sh a product on p ocess for IF the res stance of he wo nd cators (IS and IF) was compa ed mu ually and w h that of dust samples Var ous exposu e t mes we e used n s er lizer A and he chamber of the s er lizer conta ned only the test prepara ons The results are shown in Table 1

V h

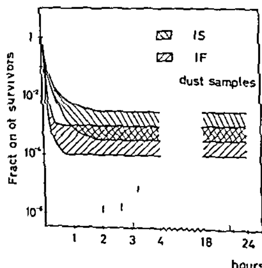
rap u y bo h ndicators to about 10 per un t af er wh ch no further inact va on could be reg s ered (F g 1) The major y of the dust samples showed growth af er exposure for 2–4 hours and even af er exposure for about 20 hours 12 showed growth out of a total of 125 spec mens

Pre hum d f ca on at 56°C gave cons derable changes n the results (Table 1) The least sens tive

S h h

showed growth in 2 out of about 150 samples af er exposure for both 4 and 20 hours

In the second ser es the res stance of IS and IF was compared mutually n s er l zer A n an ethylene ox de *procedure for rout ne d s nfect on of* var ous heat and mo sture sens t e surg cal instru ments The t me of *exposure* was *18 20 hours*



F g 1 Inact a on of the m oo gan sms n two bio og cal nd ca ors (IS and IF) and n samp es of du du ng a subop ma exposu e o e hy ene ox de (450 mg l) and wa e apou (RH 60)

It is difficult in some cases to use the reference indicator IS for control of ethylene oxide sterilization of disposable material such as syringes needles and infusion sets because its content of sea sand and wrapping material makes the indicator too bulky and water absorbent for use inside a number of medical utensils As supplement to IS Statens Seruminstitut has therefore developed a new biological indicator – IF – with the same resistance properties against the effect of ethylene oxide as IS but of smaller size and with no wrapping material around the indicator unit

In large scale gas sterilization IF is intended as an alternative to and not a substitute for IS IS is still a most suitable indicator for the control of many industrial sterilization processes

MATERIALS AND METHODS

The bacteriological preparations included in the investigations are as follows

1 Biological indicator based on spores in sand (IS)

Test strain is a *Bacillus subtilis* isolated in 1949 from straw with a view to its use in biological indicators for autoclaving and dry heat sterilization

The strain is cultivated on agar plates (beef peptone broth (1 1) NaCl 0 3% Na_2HPO_4 $2H_2O$ 0 2% agar 1 7% pH 7 3–7 4) for 4–5 days at 36–37°C When the culture contains more refractive spores than vegetative cells the layer of culture is scraped off the plates and suspended (unwashed) in physiological saline The spore suspension is poured on to sterile sand (Merck Seesand gereinigt art 7711) The wet sand is dried in vacuum and homogenized The amounts of culture saline and sand are regulated to a content of about 10^7 spores per gram dried spore sand The drying process is regulated so that the resistance to dry heat and to the combined effect of ethylene oxide and water vapour is the same as that of previous preparations The indicator unit consists of about 120 mg spore sand packed in two layers of paper The spore sand is kept in air and watertight packages from time of drying until time of use

2 Biological indicator based on spores in cotton yarn (IF)

The test strain is *Bacillus subtilis var niger* ATCC 9372 made available in 1960 by Dr *R A Hoffman* Camp Detrick Maryland U S A The strain is grown on TGY agar plates (0 5% tryptone (Difco) 0 1% d glucose (Analar) 0 3% yeast extract (Difco) 1 8% agar (Difco)) for 3–5 days at 30–32°C When the culture contains more refractive spores than vegetative cells the layer of culture is scraped off the plates and suspended (unwashed) in sterile saline Washed sterile cotton yarn is moistened with the spore suspension before vacuum drying The amounts of culture water sodium chloride and yarn are regulated to a content of about 10^6 spores per 1 cm cotton yarn The test piece consists of about 1 cm yarn

In the series of experiments described here IF was packed in two layers of paper for comparison with IS and dust samples An exception was in the fifth series which was a simulated routine sterilization of disposable medical equipment where IF was used without paper wrapping The indicator is kept protected from air and water vapour from time of drying until time of use

3 Dust samples containing about 10^5 colony forming units

The dust originated from Danish households and was collected by vacuum cleaner during the months of May June and August Three to five different households were represented in each batch of dust samples The samples were used for only a few weeks after collection Hair and coarse particles were removed and the dusts were then mixed and homogenized in mortar From time of collection to time of use the dust was protected against changes in temperature and air humidity The individual test unit consisted of about 100 mg dust packed in two layers of paper

Bacteriological methods After exposure to ethylene oxide the two biological indicators and the dust samples were cultured in fluid TGY medium pH 7 8 8 ml and 20 ml per sample respectively The two subtilis strains were incubated at 35–37°C for 14 days and the dust samples at 30–32°C for 6 weeks

Secondary culture on TGY agar plates and microscopy of stained and unstained smears were used to verify that turbidity or colonies in the medium was due to viable microorganisms and also to classify the surviving microorganisms as bacteria or fungi

Determination of the initial number of colony forming units in the indicators and dust samples was made by suspension of the specimens in physiological saline and inoculation from tenfold dilutions on to TGY agar plates In a number of the experiments cultivation of the ethylene oxide treated specimens in fluid medium was supplemented by counting the number of surviving colony forming units in parallel sets of specimens

When pre humidified specimens were used these were brought to equilibrium with high relative humidity by storage for 24 hours at 56°C ± 2°C in a little box containing an excess of water in the form of moistened cotton wool

Two sterilizers were used

Sterilizer A with a volume of 60 litres

Heating is carried out by means of a water jacket around the chamber During the whole process the temperature in the jacket is 54°C ± 1°C Transient temperature variations in the air mixture in the chamber will not be registered by the thermometer which measures the temperature in the mantle of the chamber The air is evacuated to a residual pressure of 10 mm Hg Water is admitted to 60 % relative humidity and then an ethylene oxide freon mixture (12 % ethylene oxide 44 % freon 11 and 44 % freon 12) to an ethylene oxide concentration of 450 mg per litre After exposure evacuation is carried out to below 10 mm Hg for at least one hour The time of exposure is measured from admission of the ethylene oxide freon mixture to the initiation of evacuation

TABLE 4 *Comparison of IS IF and Dust Samples in* Simulated Routine Sterilization *of Disposable Plastic and Metal Articles The Figures Shown are the Sum of the Results of Individual Experiments which Included 12 of each of the Three Bacteriological Preparations Four IF Indicator Units were placed at each of Locations A B and C*

Type of indicator		Exposure time: 4 hours: Total number of samples	4 hours: Number of samples with growth	19 hours: Total number of samples	19 hours: Number of samples with growth
Dust samples		60	9	60	2
IS		60	0	60	0
IF	Total	60	37	60	13
	At location A[a]	20	0	20	0
	At location B	20	17	20	0
	At location C	20	20	20	13

[a] Locations A B and C of indicator IF are shown in Fig 2

without needles attached The packing material for the individual items was a bag with a plastic laminate front and a paper back The multicontent packages were the original cardboard boxes In this series the advantages of the small size of IF could be utilized The pieces of cotton yarn were placed without any paper wrapping at locations where the sterilization procedure was presumed to be the least effective (Fig 2) Because of their size the indicator units of IS and the dust samples could not be placed inside the syringes These test pieces were therefore placed inside the packages for the individual items The exposure times were 4 and 19 hours in sterilizer B (Table 4)

Even the short exposure time caused inactivation of the biological indicators when located so that the gas and water vapour has reasonably easy access However nine out of a total of 60 dust samples placed similarly showed growth The indicator units placed where the gas and water vapour had difficult access showed growth from 17 out of 20 (location B) and 20 [illegible]

[illegible] inactivated In location C growth appeared from 13 out of 20 IF indicators

Incubation Period and Surviving Microorganisms

In all [illegible]

[illegible] days

The majority of the microorganisms isolated from the dust samples exposed to ethylene oxide were classified as Gram-positive rods but also fungi were observed frequently After an exposure to ethylene oxide which resulted in growth from more

TABLE 5 *Morphological Classification of Surviving Microorganisms in Dust Samples Subjected to Moderate and Severe Exposure to Ethylene Oxide Respectively*

Efficiency of exposure to ethylene oxide	Total number of strains classified	Classification of survivors as percentage of total: Bacteria: Cocci	Bacteria: Rods	Fungi
Growth from more than 90 % of the dust samples	223	8	82	10
Growth from less than 10 % of the dust samples	80	8	12	80

TABLE 2 *Comparison of IS and IF in* Routine Disinfection *in Sterilizer A (18–20 h) and in* Routine Sterilization *in Sterilizer B (4 h) The Figures Shown are the Sum of the Results of Individual Experiments with Six of each of the Two Indicators*

Procedure	Type of indicator	Total number of samples	Number of samples with growth after exposure
Routine disinfection procedure in sterilizer A	IS	84	60
	IF	84	54
Routine sterilization procedure in sterilizer B	IS	72	6
	IF	72	3

In all cases the products were heat sensitive surgical instruments with a low waterabsorbing capacity

TABLE 3 *Comparison of IS, IF and Dust Samples after Exposure to Ethylene Oxide in* Simulated Routine Sterilization *of Textiles Wrapped in Paper The Figures Shown are the Sum of the Results of Individual Experiments which Generally Included 6 or 12 of each of the Three Preparations*

Type of indicator	Exposure time: 4 hours		Exposure time: 19 hours	
	Total number of samples	Number of samples with growth	Total number of samples	Number of samples with growth
Dust samples	177	20	146	6
IS	178	72	147	0
IF	178	76	147	0

(Table 2) IS and IF gave an almost equal number of positive results

In the *third series* the resistance of IS and IF was compared in routine ethylene oxide sterilization of various heat-sensitive surgical instruments in sterilizer B The exposure time was 4 hours (Table 2) IS and IF gave almost the same number of positive results obtained in the same procedures

In the *fourth series* the resistance of IS and IF was compared mutually and with dust specimens in a simulated routine ethylene oxide sterilization where the product was cotton material wrapped in paper The indicators and the dust samples were placed in the middle of the packages and the exposure time was either 4 or 19 hours in sterilizer B (Table 3) The shorter exposure time resulted in growth almost equally often from IS and IF, and somewhat more seldom from dust samples The long exposure time resulted in growth from only a few of the dust samples

In the *fifth series*, the resistance of the two indicators was compared mutually and with dust samples in a simulated routine ethylene oxide sterilization of disposable plastic syringes with and

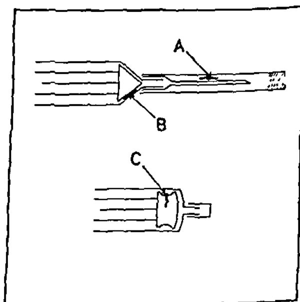


Fig 2 Location of IF in medical utensils A Between needle and protection cap B Between the conical bottom of a syringe and the plunger C Between the ribs of a rubber plunger The results of exposure to ethylene oxide (450 mg/l) and water vapour (RH 100%) are given in Table 4

other particles and the majority exist as more or less solid aggregates The number demonstrated by bacteriological determination is therefore a minimum figure The effect of other systematic errors on germ determinations acts in the same direction Only the organisms which are given suitable growth conditions will be detected For instance obligate anaerobic organisms are not registered in this study

However, despite the shortcomings dust from private dwellings was chosen because 100 mg of this natural product has about the same volume and contains the same number of microorganisms as one biological indicator unit It is our experience that dust collected from vacuum cleaner or air filter in areas for production of medical equipment contains a smaller number of colony forming microorganisms per gram (2 9) Due to the larger samples necessary to obtain sufficient numbers of microorganisms such samples may provide too severe an obstruction against ethylene oxide and water vapour as compared with that of the biological indicators

The microbial contamination of disposable equipment cannot be used as test material because the initial contamination per unit is too small in relation to the sterilization standard Irrespective of how the experiments are set up extrapolation from small to large inactivation factors will be necessary and we wish to avoid extrapolation because the inactivation curve for mixed microbial populations in natural products cannot be considered to be sufficiently elucidated

It has been stated (8) that the indicator IS has an unrealistically high resistance since it contains endospores enclosed in sodium chloride crystals *Hess et al* (8) showed that the number of highly resistant germs was less than one per 10^7 in dandruff and less than one per 10^5 and one per 10^4 respectively in two different samples of airborne contamination from production areas If these results are compared with the general experience from initial countings on different disposable articles before sterilization and with the Scandinavian standard for sterilized medical equipment where the relevant inactivation factors are between 10^6 and 10^9 it will be seen that the figures cannot elucidate the relevance of the resistance of IS *Hess et al* demonstrate that the inactivation curve for microorganisms in the pollutions is different from that for the spores in the indicator However a biological indicator serves its purpose when it gives growth after exposure to a sterilization procedure which is insufficient as compared to the chosen standard for sterilized products and no growth when the efficiency of the procedure is as good or better than desired Resistance characteristics for an indicator giving a parallel to the inactivation curve for the microorganisms on and in the medical products would be difficult to design and very difficult to standardize and would result in the inaccurate test results which are unavoidable when the test samples follow the typical downward convex inactivation curve for a mixed population of microorganisms in natural contamination

The fifth series of experiments showed the well known phenomenon that the location of the biological indicator in the product to be sterilized may be decisive for the value of this type of control In addition they provided justification for *Rubbo & Gardner's* (15) mistrust of ethylene oxide sterilization of injection syringes and infusion sets In our opinion the warning given by *Rubbo & Gardner* has not received sufficient attention outside the Scandinavian countries When the design of the products makes ethylene oxide sterilization questionable gamma or electron irradiation should be adopted for sterilization of disposable medical equipment (2)

Classification of the microorganisms isolated from dust samples after exposure to ethylene oxide gave the anticipated result that when the probability of organisms surviving was relatively great rapidly-growing Grampositive bacteria were found frequently If they survive such robust strains will often dominate in the culture after exposure to ethylene oxide even though there may also have been other viable cells present in the dust samples When the frequency of surviving organisms is relatively small there will probably be no or only one or a few viable cells left in the dust samples Therefore competition between different organisms will be infrequent It is notable therefore that it is fungi which are the organisms most often demonstrated in dust samples subjected to the most effective inactivation procedures The same phenomenon has been observed previously (9) in dust samples from production areas in Denmark

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than 90% of the dust samples sporeforming rods dominated Where the exposure was so efficient that less than 10% of the dust samples showed growth fungi were the most frequent finding (Table 5) This distribution would indicate that the conditions after exposure favoured the growth of Gram positive sporeforming rods

DISCUSSION

The biological indicator IF has been designed for insertion into items of disposable medical equipment The indicator must be placed in the product to be sterilized at locations which are the most difficult for water vapour and ethylene oxide to reach

The indicator strain is that most frequently used for microbiological control of ethylene oxide sterilization (1 5 16) and is easily recognizable and harmless – if any microorganism can be regarded as harmless in this connection

Differences in the products to be sterilized and in the sterilization procedures used will inevitably cause differences in the results when the resistance of the two indicators is compared in relation to various practical applications of biological indicators Therefore the results given in this study can only be examples The first series of experiments showed that IF more readily comes into equilibrium with the relative humidity in the environments than IS However the other experiments showed that this difference was not significant when the amount of water vapour available was large

When only small amounts of water are available in the micro environments the inactivation curve of the microorganisms in both indicators is different from that of organisms in the natural product chosen for comparison in this study (Fig 1) After an exposure which resulted in about 10% of the dust samples showing growth corresponding to an inactivation factor of 10^5–10^6 for the microorganisms in the dust samples the inactivation factor for the spores in both indicators is only 10^3–10^4 There are about 100 times as many protected microorganisms in the biological indicators as in the dust samples Protection of the spores in the indicators is provided by sodium chloride crystals and this ceases if the humidity in the micro environment is high enough to disorganize the crystals The biological indicators are therefore very sensitive to deficiencies in the distribution of water in the sterilization procedures The change from protected to unprotected state is a fast process when enough water is available A small number of protected spores per indicator unit causes a large number of ambiguous test results with growth after exposure from some though not all indicator units However it is essential for the practical value of a biological indicator that this twilight zone where the change from growth from all samples to no growth takes place is narrow The large content of protected spores in the biological indicators will ensure that clear warning is given if a sterilization process has not been sufficiently effective to inactivate the protected microorganisms

It is our opinion that the comparisons between the resistance of the indicators and that of the microorganisms in dust samples confirm that the resistance of IF and IS is regulated to a realistic level The experiments in which it was possible to include dust samples showed that there is a small fraction of microorganisms in dust which are difficult to inactivate with ethylene oxide and water vapour under conditions resembling sterilization in practice Exposures resulting in growth from no or one indicator units per 100 units give growth from one or a few dust samples out of each 100 samples tested corresponding to an inactivation factor of 10^6–10^7 An inactivation factor of this size must be regarded as adequate for sterilization of a number of disposable articles with small numbers of organisms per unit before sterilization However it does not provide any extra margin of safety if the Scandinavian standard for sterilized medical products is to be adhered to (10 12 14) The standard requires that the number of colony forming microorganisms per one million sterilized product units must not exceed one The comparisons also show that there is no simple relationship between the inactivation factor for the test strain in the biological indicator and the inactivation factor for a mixed population of microorganisms in a natural product such as dust

As an example of microbial contamination the dust specimens chosen are not without shortcomings The resistance of the microorganisms in the dust can only correspond to that of microbial contamination under conditions resembling those i Denmark The climate living conditions and customs of the population must have a great influence on the number type and resistance of contaminating microorganisms We chose to carry out the comparisons with dust samples collected in months during which the heating of the houses had only little influence on the resistance of the microorganisms in dust However in countries with a warmer and less humid climate than i Denmark the example is of less value Furthermore the number of organisms demonstrated per dust sample and the number of dust samples showing growth are influenced by the investigation methods The organisms in the dust are bound to

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Acta path microbiol scand Sect B 87 155-160 1979

DIAGNOSIS OF POSTNATAL RUBELLA BY THE ENZYME-LINKED IMMUNOSORBENT ASSAY FOR RUBELLA IgM AND IgG ANTIBODIES

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Vejtorp M Fanøe E & Leerhoy J Diagnosis of postnatal rubella by the enzyme linked immunosorbent assay for rubella IgM and IgG antibodies Acta path microbiol scand Sect B 87 155-160 1979

A semi-automated enzyme-linked immunosorbent assay (ELISA) was established for the detection of rubella IgM antibodies in non fractionated sera A cut off level between rubella IgM positive and negative sera was determined by a study of sera without rheumatoid factor from 200 blood donors Testing of 12 donor sera containing rheumatoid factor showed that 5 sera gave a positive result in the rubella IgM assay Rubella IgG and IgM antibodies were quantified by ELISA in a study of 214 serial serum specimens drawn from 16 patients with rubella during a period of up to 10 years after the infection Peak values of the IgM antibodies were reached approximately 8 days after onset of the rash and the persistence of the IgM antibodies ranged from 17-90 days with the exception of one patient with a prolonged IgM response The rubella IgG antibodies increased slowly after the rash and reached maximum levels about 50-120 days after which a minor decrease was observed The results of the present study indicate that ELISA is suitable as a routine procedure for the serodiagnosis of recent rubella

Key words Enzyme immunoassay rheumatoid factor

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Determination of specific IgM antibodies is the method of choice for the diagnosis of recent rubella when acute phase sera are lacking or when a rapid diagnosis based on study of only one blood specimen is required

Haemagglutination inhibition (HI) tests (2 25) or the indirect immunofluorescence technique (5) applied on serum fractions obtained by ultracentrifugation are commonly employed for this purpose Recently *Meurman et al* (13) described a sensitive and reliable radioimmunoassay (RIA) for the detection of rubella IgM antibodies without preceding separation of the immunoglobulins However in general RIA is impeded by the cost of reagents and equipment and the disadvantage of handling radioactive isotopes

A simple inexpensive and reliable enzyme linked immunosorbent assay (ELISA) for the routine detection of rubella IgM antibodies is described here This assay and an ELISA for quantification of rubella IgG antibodies recently published by *Vejtorp* (22) were employed in a study of rubella IgG and IgM antibodies in a total of 214 serial serum specimens obtained from 16 patients with rubella during a period of up to 10 years after the infection

MATERIALS AND METHODS

Serum Specimens

From 16 patients (12 females and 4 males) with acute rubella infections a total of 214 sera were drawn over a period of from 4 days before to 3 684 days after the rash

The clinical diagnosis of rubella was confirmed by a more than fourfold rise in HI titre of paired sera (15 patients) or by demonstration of rubella IgM antibodies by the HI test after rate zonal ultracentrifugation (1 patient). In addition, sera from 212 blood donors were tested for rubella IgM antibodies by ELISA. All sera were tested in duplicate. Positive and negative reference sera were included in each experiment.

ELISA Procedures

Rubella IgG antibodies were determined as described previously (22). The ELISA for determination of rubella IgM antibodies was a modification of this procedure. Briefly, microtitre plates (Cooke M129, Dynatech) were used as solid phase for rubella virus and control antigen. The protein content of the undiluted virus and control antigens determined by spectrophotometry at 280 nm was approximately 100 mg/l. After incubation with diluted serum the plates were washed in an automatic plate washer (Dynatech® AM 71). The specific immunoglobulins were detected by the addition of a commercial alkaline phosphatase conjugated anti-IgG (γ-chain specific) and − IgM (μ chain specific, ORION diagnostica). After addition of p nitrophenyl phosphate and an incubation time of 35 min the reaction was interrupted and the colour intensity read on a spectrophotometer with a rapid sampling microcuvette (Gilford Stasar II).

The results were expressed as an E-value which was the difference between the absorbances obtained by testing the sera with virus and control antigen. The absorbance of the positive reference sera was in each experiment adjusted to the same values in order to reduce between assay variations.

Rate Zonal Ultracentrifugation

The serum immunoglobulins were separated by rate zonal ultracentrifugation as described previously (24). The IgG and IgM contents of the serum fractions were measured by single radial immunodiffusion (11) with plates containing anti IgG (γ chain specific) and anti IgM (μ chain specific) respectively.

IgM Rheumatoid Factor

The IgM rheumatoid factor was quantified by the method described by Vejtorp et al (23). An E value exceeding the 95th percentile in a previous study of sera from 400 blood donors was regarded as indicative of the presence of rheumatoid factor.

Statistical Methods

The results of the study of rubella IgM antibodies in sera from male and female blood donors were compared by the Students t test.

RESULTS

Determination of the Optimal Conditions of the Rubella IgM Antibody Assay

The highest ratio between the E-values of IgM positive and negative sera was found by a dilution of the virus and control antigens of 1/20, a serum dilution of 1/200, and by incubation on a water bath at 37° C for 2 h for the diluted serum samples and for 3 h for the diluted conjugate. The non-specific binding of the anti IgM conjugate was reduced further by use of 10% (v/v) normal swine serum (DAKO-immunoglobulins Ltd) in phosphate buffered saline-tween for dilution of the conjugate.

Immunoglobulin Class Specificity

The immunoglobulin class specificity of the conjugates was tested by studying immunoglobulins separated by rate zonal ultracentrifugation of an acute phase serum (Fig 1). Positive values of the IgG and IgM assays were restricted to the fractions which contained IgG and IgM antibodies, respectively.

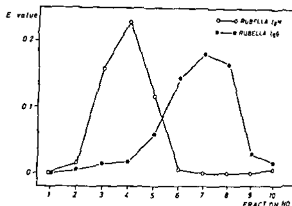


Fig 1 An acute phase serum from a patient with rubella tested by ELISA for rubella IgM and IgG antibodies after separation by rate zonal ultracentrifugation.

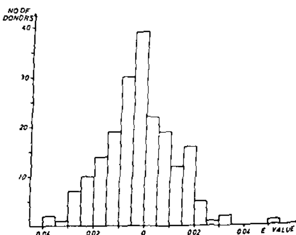


Fig 2 Sera from 200 blood donors tested by ELISA for rubella IgM antibodies.

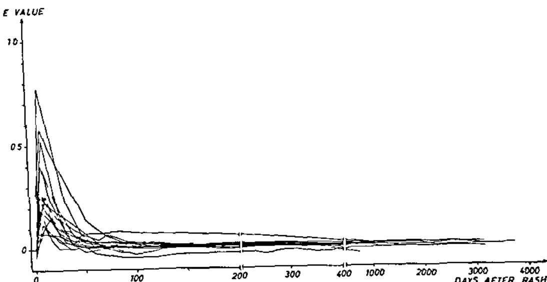


Fig 3 Rubella IgM antibodies determined by ELISA in 214 serial serum samples from 16 patients with rubella

IgM Rheumatoid Factor Determination

RF was not detected in the sera from the 16 patients with rubella whereas 12 of the 212 donor sera contained significant amounts of RF

Rubella IgM Antibody Determinations

The results obtained by testing sera from 200 blood donors (100 males and 100 females) without RF can be seen in Fig 2 There was no significant difference between the E values of the sera from male and female donors ($p > 0.20$) A cut-off level between rubella IgM negative and positive sera was set at the arithmetic mean plus three standard deviations corresponding to an E value of 0 039 The E value of one of the donor sera exceeded that level The activity in ELISA after rate zonal ultracentrifugation of this serum was located to the IgM fractions Also the E values of 5 of the 12 donor sera with RF exceeded 0 039

The IgM antibody response in 16 patients with rubella is shown in Fig 3 IgM antibodies were not detected in the samples drawn on the day of the rash or in a sample collected 4 days prior to the rash whereas 5 of 8 samples taken 1–2 days after onset of the rash contained rubella IgM The maximum E values which were observed about 8 days after appearance of the rash showed large individual differences These declined corresponding to a half life of the IgM antibody concentration varying between 4–25 days (mean 10 days) with a prolonged IgM response Rubella IgM was detected in a serum sample drawn from this patient 196 days after appearance of the rash but not in a subsequent sample drawn after 576 days (Fig 4) As regards the other patients the persistence of rubella IgM antibodies ranged from 17–90 days and the IgM antibodies disappeared from the sera of half the patients about 50 days after the rash The E values of all sera drawn more than 196 days after the rash did not exceed 0 016

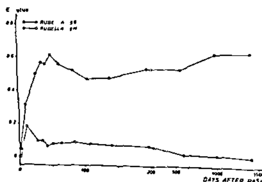


Fig 4 Rubella IgM and IgG antibodies determined by ELISA in serial serum samples from a patient with a prolonged IgM antibody response after rubella

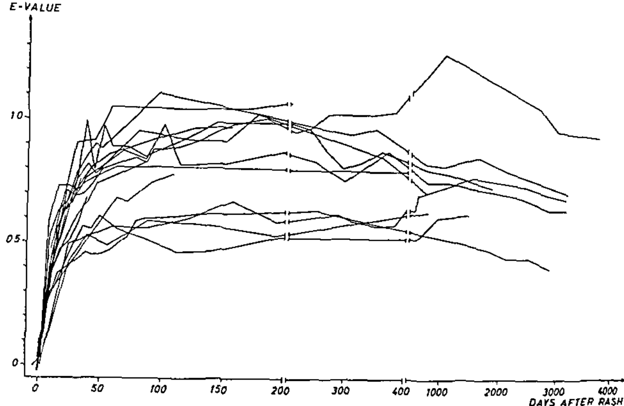


Fig 5 Rubella IgG antibodies determined by ELISA in 214 serial serum samples from 16 patients with rubella

Rubella IgG Antibody Determinations

Rubella IgG antibodies were not detected in samples drawn during the first 3 days after the rash, whereas all sera collected later contained rubella IgG (Fig 5) After a slow rise in the E-values a plateau was reached about 50–120 days after onset of the rash This was followed by a minor decline during subsequent years A new rise in the E-values of approximately 20 % was observed in sera from 3 patients 1–4 years after the infection The IgG antibody level of the patient with prolonged IgM response remained low until after disappearence of the IgM antibodies (Fig 4)

DISCUSSION

An ELISA for the detection of IgM class specific antibodies to rubella virus was introduced recently by *Voller & Bidwell* (27) who described the detection of rubella IgM antibodies with ELISA in sera from one of two persons vaccinated against rubella A similar assay has been used for the determination of IgM antibodies against cytomegalovirus (1, 3, 18) and against bacterial antigens (9, 21)

A major problem of the IgM antibody assay is the high background level of E-values caused by non-specific binding of IgM or conjugate This was compensated for in the present assay by subtraction of the values obtained by testing with the control antigen from the values obtained by testing with virus antigen Also a meticulous assessment of optimal temperatures, incubation times, and concentration of the components of the assay was necessary in order to reduce the non specific background levels

Another problem connected with this assay is false possitive results caused by the presence of RF, which is an anti-IgG of IgM class RF might attach to rubella IgG bound to the virus antigen and be detected by the anti IgM conjugate Such false positive results have been observed by the study of rubella IgM antibodies with the RIA (14) and the immunofluorescence technique (6, 19) Consequently, sera with rubella IgM antibodies detected by ELISA should be tested for RF, which, when present, might be removed by absorption with aggregated IgG (19) or with IgG bound to an immunosorbent (10) Activation of RF is, however, not common during rubella infections (14) This was confirmed in the present study by the absence of significant amounts of RF in all sera from the 16 patients with rubella

The influence of IgM RF and rubella IgG on the false positive rubella IgM result will need further

clarification This applies also to a possible mutual interaction of rubella IgG and IgM antibodies in the assay

A study of sera from 200 donors was used for the establishment of a cut-off level for the E values of rubella IgM negative and positive sera (Fig 1) Generally low E values were found The E value of one of the donor sera was in the lower part of the range of IgM positive sera. The activity of this serum in ELISA was located to the IgM fractions which might be explained by non specific binding or more probably by a recent rubella infection The relevance of the chosen limit for the E value between rubella IgM positive and negative sera viz 0 039 was illustrated by the observation of [illegible]

[illegible]

antibodies is in agreement with earlier findings (4 8 15 16 26) A prolonged IgM antibody response after rubella has previously been observed in studies of the rubella IgM antibodies with the immunofluorescence technique (7) the haemagglutination inhibition test (20) and with a RIA (12) Thus *Meurman* (12) observed a prolonged persistence in 2 out of 49 cases of acute rubella. A prolonged rubella IgM antibody response has been reported after rubella complicated by thrombocytopenic purpura carpal tunnel syndrome and arthritis (7 12 17) but infrequently after uncomplicated infections (12)

The rise of the E values determined by the rubella IgG antibody assay proceeded slowly after the infection and a plateau was reached 50–120 days after the rash This permits a diagnosis by demonstration of a significant rise in the E values also when the first serum specimen was drawn as late as 10–20 days after onset of the rash The rise in the E values observed 1–4 years after rash in sera from 3 patients was not accompanied by reappearence of the rubella IgM antibodies and could possibly be interpreted as reinfection

The results of the present study demonstrate the potential of ELISA for the diagnosis of recent rubella by study of the specific IgG and IgM antibodies The ease of performance of a semiautomated assay will probably result in an increasing use of ELISA as a routine diagnostic procedure

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SEROEPIDEMIOLOGY OF H1N1 INFLUENZA: STRIKING DIFFERENCES IN THE ATTACK RATE AMONG YOUNG PEOPLE

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Pyhälä R Aho K & Visakorpi R Seroepidemiology of H1N1 influenza striking differences in the attack rate among young people Acta path microbiol scand Sect B 87 161–164 1979

The H1N1 influenza outbreak in winter 1977–78 covered the whole of Finland in a uniform fashion whereas striking differences emerged between various population segments in the susceptible age group The attack rate was 68 per cent among young servicemen who had been in the armed forces during the epidemic and 33 per cent among those who had entered the service after the epidemic but only 6 per cent among pregnant women of the same age Based on routine specimens sent to the laboratory for reasons unrelated to influenza the attack rate was 4 per cent among children born in 1973–76 and increased by age up to 45 per cent among subjects born in 1957–6? The findings on pregnant women and small children suggest a novel mechanism of innate resistance operating specifically against H1N1 influenza

Key words Influenza A (H1N1) seroepidemiology pregnancy

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Serological surveys providing information on the occurrence of antibodies against influenza are of value in assessing the size of epidemics and in predicting new influenza outbreaks The Finnish influenza programme includes the yearly evaluation of pre-epidemic antibody levels in serum samples of people in different age groups (6 8) and the testing of serial blood specimens from Rh negative pregnant women (7) In addition influenza investigations have been carried out in the Finnish Defence Forces in a uniform fashion since 1972 (9)

In winter 1977–78 a »new« subtype of influenza A virus H1N1 appeared and spread to most countries in the world (2) Children over 5 years of age and young adults were mainly infected (12) Formerly when new influenza A subtypes had emerged the preceding antigenic subtype rapidly disappeared but now the H3N2 variants continued to circulate in the community The »new« virus closely resembled the H1N1 influenza viruses of 1947–57 People born after 1956 as a rule lacked the haemagglutination inhibiting (HI) antibodies against the epidemic virus before the outbreak (6) their presence in postepidemic specimens was therefore indicative of infection This offered additional scope for the assessment of infection rate

The H1N1 outbreak reached greater dimensions in Finland than in most other western countries We observed that the epidemic swept the country in a uniform way whereas striking differences were noted in the attack rate among various segments of young people some of them quite unexpected These findings are reported in the present communication

MATERIALS AND METHODS

Based on cases confirmed virologically at the Central Public Health Laboratory the outbreak by H1N1 influenza commenced during the first weeks of January

1978 and terminated in the beginning of March. The outbreak by the H3N2 virus had its onset a few weeks later and 80 per cent of the positive findings had been made before the 16th of March.

Postepidemic blood samples were collected in May–June 1978 at three military training centres from two groups of servicemen born in 1957–61

a) from those who had been in service during the H1N1 epidemic and
b) from those who had entered the service after its termination.

Serial blood specimens from Rh negative pregnant women sent to the laboratory were stored and used for influenza investigations. The pre-epidemic specimens were collected within the period from 16th September to 31st December 1977 and the postepidemic specimens from 16th March to 30th June 1978. A great majority of the subjects were at the second trimester of pregnancy during the influenza outbreak.

Routine specimens sent to the laboratory for reasons unrelated to influenza were collected within the same period as the second specimens from pregnant women. The specimens were obtained from general hospitals in different parts of the country. This collection of sera was stored at 4°C and the others at −20°C. The principles presented by *Robinson & Dowdle* (10) were followed in HI tests (7) performed in June–September 1978. The specimens tested simultaneously contained representatives from all four collections and the paired specimens from the same subjects were always tested simultaneously. The sera were pretreated with cholera filtrate (Philips Duphar B.V. Holland) to remove non-specific inhibitors. Infected allantoic fluids from embryonated eggs diluted to contain 4 HA units of virus were used as antigens. The virus strains were as follows: influenza A/Finland/30/77 (H1N1), A/Finland/23/75 (H3N2) and A/Finland/61/78 (H3N2).

RESULTS

The occurrence of postepidemic HI antibodies was studied in servicemen at three military training centres (Table 1).

Antibodies against the H1N1 virus were detected in 65–76 per cent of those who had been in service during the H1N1 epidemic and in 31–35 per cent of those who had entered the service later on. For the sake of comparison the sera were also tested against one (A/Fin/23/75) of the two closely related epidemic strains of the H3N2 subtype. No corresponding difference was observed; in fact antibodies occurred slightly more frequently among those who had entered the service after the epidemic.

The servicemen who had entered the service after the epidemic were from 9 of Finland's 11 provinces. No appreciable differences in the occurrence of antibodies against the H1N1 virus were noted between the 9 provinces indicating that the epidemic had swept in a uniform fashion over at least most parts of the country.

We have used serial blood specimens from Rh negative pregnant women for influenza investigations since 1971. The rate of infection during the six previous epidemics caused by H3N2 variants of influenza virus had varied from 27 per cent (in 1971–72) to 14 per cent (in 1976–77).

In winter 1977–78 the attack rate by the H3N2 virus was 8 per cent (Table 2). The corresponding rate by the H1N1 virus was 6 per cent among women born in 1956 or after and less than 1 per cent among those born before 1956.

All the pregnant women born in 1956 or after

TABLE 1. *Postepidemic Antibodies to H1N1 and H3N2 Subtype Viruses in Servicemen from Three Training Centres*

	Military training centres	Seropositive[a)] subjects: those who had entered the service before the outbreak	after the outbreak
Antibodies to A/Fin/30/77 (H1N1)	A	57/ 75 76%	53/150 35%
	B	104/161 65%	49/152 32%
	C	103/150 69%	47/150 31%
Antibodies to A/Fin/23/75 (H3N2)	A	18/ 75 24%	56/150 37%
	B	53/161 33%	68/152 45%
	C	45/150 30%	48/150 32%

a) A titre of ≥ 12

TABLE 2 *The Rate of Infection with H1N1 and H3N2 Subtype Viruses in Pregnant Women*

Year of birth	Rate of infection[a)]	
	H1N1	H3N2[b)]
1935–55	4/565 0.7 %	46/565 8 %
1956–60	5/ 86 6 % [c)]	6/ 86 7 %

a) A ≥ 4 fold increase in antibody titre

b) Two virus strains A/Finland/73/75 (an A/Victoria like strain) and A/Finland/61/78 (an A/Texas like strain) were used

c) The rate of infection remained the same when a titre of ≥ 12 in the postepidemic serum was used as a criterion

lacked the pre-epidemic HI antibody against the H1N1 virus. This gives support to previous findings (6) and indicates that the attack rate among young people can be assessed on the basis of a single specimen taken after the epidemic. The four older subjects with serologically verified infection also lacked the pre-epidemic HI antibody.

The 6 per cent attack rate by the H1N1 virus observed among pregnant women was low compared with the observations on young healthy males (Table 1) and also in contrast with the diagnostic findings (virus isolations and significant rises in antibody titre) from specimens sent to the laboratory from patients with respiratory infections. In young age groups (patients born in 1956 or later, specimens from military units excluded) 31 out of the 35 positive findings were indicative of H1N1 infection and only 4 findings of H3N2 infection.

The occurrence of HI antibodies against the H1N1 subtype virus was also investigated in postepidemic specimens sent to the laboratory for reasons unrelated to influenza (Table 3). Only rarely were antibodies detected (in 4 per cent) among small children born in 1973–76. Their frequency increased up to 45 per cent among the subjects born in 1957–62 with slight excess among males.

DISCUSSION

The absence of any pre-epidemic HI antibody prior to the H1N1 outbreak in winter 1977–78 in young people provided a unique opportunity for the assessment of the attack rate using single serum specimens taken after the outbreak. In order to obtain some information on the occurrence of infection as related to the degree of exposure we compared two groups of servicemen, those who had been in service during the epidemic and those who had entered the service after it. The attack rate proved to be twice as high in the former group.

We were also interested in extending our previous observations (7) on the relationship between resistance against influenza and the pre-epidemic level of serum HI antibody to cover a new subtype of influenza virus. To our surprise the infection rate by the H1N1 virus was quite low among pregnant women, at most one fifth of the rate expected on the basis of the infection rate among other people in the same age and on the figures concerning the H3N2 infection. The infection rate was also low in children under 5 years of age and increased gradually by age up to a ten fold rate among young adults. This same finding concerning the age relationship was obviously made by *Zhdanov and co-workers* (12) although they mention it only briefly without giving any details. Corresponding observations were not made in connection with the H3N2 epidemics.

It is tempting to link together the findings on pregnant women and on small children in spite of the difficulty of presenting a meaningful hypothesis to explain them. It may be worth recalling that host factors such as age and sex are important determinants in many diseases known or suspected to be

TABLE 3 *Postepidemic Antibodies to H1N1 Subtype Virus in Blood Specimens from General Hospitals*

Year of birth	Seropositive[a)] subjects		
	male	female	total
1957–62	26/49 53 %	23/60 38 %	49/109 45 %
1963–67	15/37 41 %	15/45 33 %	30/ 82 37 %
1968–72	10/63 16 %	6/36 17 %	16/ 99 16 %
1973–76	1/38 3 %	2/31 6 %	3/ 69 4 %

a) A titre of ≥ 12

infectious in origin Thus infectious mononucleosis as a clinical disease characteristically affects adolescents and young adults although the EB virus antibody surveys clearly indicate that most people have already been infected before puberty Further toxoplasmic lymphadenopathy serves as an example of an infectious disease with a marked female predominance (*1*)

Some viral infections appear to be increased in severity during pregnancy and increased maternal susceptibility to primary infection or reactivation of latent infection could both occur as a result of depressed T cell function (5) To our knowledge no one has reported any protective effect exerted by pregnancy against an infection

Murine models have shown that both genetic factors (4) and age (3) play a role in the resistance against influenza and in man there is some evidence of HLA associated genetic resistance operating against this infection (11) We reported in the present communication that small children and pregnant women are only rarely infected by the »new« H1N1 influenza virus although they lack the pre epidemic HI antibody and any previous experience with the virus The findings suggest a novel mechanism of host parasite relationship peculiar to the H1N1 virus It remains to be seen if the virus will be capable of adapting to the human host in such a way that it can overcome the restrictions mentioned above

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IMMUNOCHEMICAL ANALYSIS OF THE TEICHOIC ACID FROM *STAPHYLOCOCCUS HYICUS*

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Osland A Grov A & Oeding P Immunochemical analysis of the teichoic acid from *Staphylococcus hyicus* Acta path microbiol scand Sect B 87 165–169 1979

The wall teichoic acid of *Staphylococcus hyicus* has been isolated and characterized The teichoic acid is a glycerol phosphate polymer with glycosidically linked N acetylglucosamine Interaction with concanavalin A and susceptibility to α but not to β N acetylglucosaminidase showed that the sugar is in the α-configuration

Key words *Staphylococcus hyicus* teichoic acid immunochemistry

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In 1950 *Sompolinsky* described a Gram positive coccus isolated from exudative epidermitis in pigs which he named *Micrococcus hyicus* (21) It was later realised that this bacterium was not a member of the genus *Micrococcus* and the name *Staphylococcus hyicus* came into general use in veterinary publications Strains isolated from the skin and the

its separation into two subspecies *i e hyicus* and *chromogenes* is proposed (7)

Agar precipitation for the serological demonstration of the wall teichoic acids revealed that all strains of *S hyicus* ss *hyicus* and ss *chromogenes* produced a precipitation line not previously identified The substance responsible for this line was provisionally designated poly A1 (6 7) The present report describes the isolation of the teichoic acid of *S hyicus* as well as its chemical and serological characterization

MATERIALS AND METHODS

Strain

S hyicus ss *hyicus* NN 308 was supplied by Dr *L A Devriese* (Gent Belgium) The strain had been isolated from epidermitis lesions in a pig The bacteria were grown in nutrient broth consisting of 15 g proteose peptone 2 5 g liver digest 5 g yeast extract (all from Oxoid England) and 5 g NaCl per litre medium Bacteria were grown for 6 h in 200 ml Erlenmeyer bottles containing 100 ml medium These cultures were then transferred to 2 l Erlenmeyer bottles containing 1 l medium and the bacteria were grown for 18 h Cultivation was performed at 37° C in a rotary shaker at 125 rev/min The cells were harvested by centrifugation at 10 000 × *g* for 30 min

Standard Teichoic Acids (Polysaccharides)

Poly Aβ (β N acetylglucosaminyl ribitol teichoic acid) from *Staphylococcus aureus* Wood 46 poly Bx (α glucosyl glycerol teichoic acid) from *Staphylococcus epidermidis* 1254 and poly C (β N acetylglucosaminyl glycerol teichoic acid) from *Staphylococcus saprophyticus* 3519 were as earlier described (19)

Isolation of Polysaccharide

The polysaccharide of *S hyicus* tentatively called poly A1 was extracted from whole bacteria with 70 mM phosphate buffer pH 6 5 at 37° C Acid and ethanol precipitation was carried out as described earlier (12) The polysaccharide was then purified on a DEAE cellulose col mn (14

refractive index using a Laboratory Data Control refractometer

Poly A1 was also isolated by the extraction of a cell wall preparation (9) using 10 per cent TCA at 4° C overnight This polysaccharide was used for the determination of possible ester linked D alanine

Acid Hydrolysis

Samples (2–5 mg) of polysaccharide were hydrolysed with 0 5 ml 3N HCl for 3 h at 100° C or 6N HCl for 20 h at 105° C in sealed tubes flushed with nitrogen The HCl was removed by evaporation to dryness *in vacuo* and the residues were washed with water after which chromatographic analysis was performed

Qualitative and Quantitative Analyses

Circular and descending paper chromatography was carried out on Whatman No 1 paper with the following solvent systems

A Butanol Acetic acid H_2O (4 1 1 v/v)

B Propanol NH_3 H_2O (6 3 1 v/v)

C Ethylacetate pyridine H_2O (40 11 6 v/v)

The detecting reagents were ninhydrin (amino acids and amino sugars) alkaline silver nitrate (sugar alcohols reducing sugars and amino sugars) (17) sodium periodate benzidine (sugar alcohols) (4) and perchloric acid ammonium molybdate-H_2S (phosphate esters) (10) Preparative paper chromatography was carried out on Whatman 3 MM paper The determination of carbohydrates and sugar alcohols as acetylated derivatives of the corresponding alditols was carried out using a Hewlett-Packard gas chromatograph with glass columns (0 2 × 200 cm) packed with 3 per cent poly A 103 coated on Supelcon AW DMCS 100/120 mesh (Supelco Inc USA)

The quantitative determination of amino acids as trifluoro acetylated butyl esters (20) was performed by gas liquid chromatography using a Model 900 Perkin Elmer with glass columns (0 175 × 185 cm) packed with 0 325 1 5 5 (ethylene glycoladipate) coated on Chromosorb WAW HT80/100 mesh (Supelco Inc)

Hexosamines were determined quantitatively by a modified Elson Morgan method (15) and phosphorus as described in (22) The presence of ester linked D alanine was examined by paper chromatography of a 1N NH_4OH hydrolysate of the TCA extracted polysaccharide

Enzymatic Treatment

N acetylglucosaminidases A crude preparation of an enzyme containing both α and β N acetylglucosaminidase activity was obtained from the limpit *Patella vulgata* by homogenization and acid precipitation as described in (3) The assay mixture contained 100 mM sodium citrate buffer pH 4 0 2 mM O nitrophenyl N acetylglucosaminid (α or β) (Koch Light Ltd England) and varying amounts of the enzyme the total reaction volume was 1 ml The reaction was carried out for from 10 to 30 min at 37° C The reaction was terminated by adding 2 ml of 0 25 M sodium carbonate The liberated O nitrophenol was measured spectrophotometrically at 400 nm One unit of N acetylglucosaminidase activity is defined as the number of μmoles of O nitrophenol released per ml per min The relative amounts of α and β-activity of the enzyme preparation was found to be 1 to 5, respectively

Polysaccharides (0 1 mg/ml) were treated with the enzyme (0 01 α units/ml) in 100 mM sodium citrate buffer pH 4 0 at 37° C for 40 h After treatment the reaction mixture was neutralized and then double diffusion was performed

β N acetylglucosaminidase was obtained from Boehringer (West Germany) The activity was measured and polysaccharides were treated as described for the N acetylglucosaminidase from *P vulgata* except that 100 mM sodium citrate buffer pH 4 4 was used Digestion of polysaccharides was performed using 0 03 β units/ml

Phosphatase Test samples were treated with alkaline phosphatase (Type II Sigma USA) in 10 mM ammonium carbonate buffer pH 9 3 for 12 h at 37° C (2) The enzyme was precipitated by using ethanol and removed by centrifugation The supernatant was evaporated to dryness and examined by chromatography

Serological Methods

Immune sera were raised against formalin killed bacteria by intravenous injections (18) into New Zealand white rabbits of the Department s breed Double diffusion in agar was performed as described previously (11) Reaction of polysaccharide with concanavalin A (Con A) (Sigma) was carried out in 1 per cent agar the concentrations of Con A and polysaccharide being 10 mg/ml and 2 mg/ml respectively Immunoelectrophoresis was performed as described earlier (14)

RESULTS

Purification of Polysaccharide

Polysaccharide A1 was obtained by four extractions of 100 g cells (wet weight) followed by purification on DEAE-cellulose and Sephadex G-100 The yield was 200 mg polysaccharide Poly A1 was eluted from the DEAE-cellulose column at a molarity of KCl between 0 27 and 0 36 M The K_{av} was 0 3 when poly A1 was eluted from Sephadex G-100 Desalted and lyophilized polysaccharide dissolved in distilled water at a concentration of 1 mg/ml had an OD at 280 nm and 260 nm of 0 025 and 0 04 respectively thus indicating little protein and nucleic acid contamination

Chemical Analysis

Chromatographic analysis of 3N HCl hydrolysates in solvent system C and B showed the presence of N acetylglucosamine and glycerol No ribitol could be detected These findings were confirmed by gas chromatographic analysis

Circular paper chromatography in the solvent system B showed the presence of glycerol and

glycerol 1 phosphate as detected by periodate benzidine spray

Preparative paper chromatography of hydrolysates was performed and the spot corresponding to glycerol 1 phosphate (compared to standard) was cut off and the sample eluted with water The sample was then treated with alkaline phosphatase and subjected to paper chromatography Spraying with periodate benzidine showed the presence of free glycerol only

Chromatograms of 3N HCl hydrolysates developed in the solvent system B and sprayed with the molybdate reagent showed only two spots one major spot corresponding to glycerol 1 phosphate and a second weak spot with R_f = 0 12 corresponding to glycerol diphosphate (16) No sugar phosphate could be detected

Glucosamine was identified by paper chromatography of hydrolysates in solvent systems A and C detected by the ninhydrin and alkaline silver nitrate reagents No other sugar or aminosugar could be detected

The amounts of phosphorus and N acetylglucosamine obtained by colorimetric analysis were 4 0 per cent and 27 per cent respectively the molar ratio of N acetylglucosamine to phosphorus thus being 0 92 to 1

Paper chromatography of 6N HCl hydrolysates *in solvent* system A showed the presence of the usual peptidoglycan amino acids *vi* alanine glycine glutamic acid and lysine Quantitative analysis by gas chromatography showed 0 13 0 25 0 052 and 0 055 µmoles/mg respectively

The amount of free glycerol *in* 6N HCl hydrolysates obtained by gas chromatographic analysis was 9 7 per cent the molar ratio of glycerol to phosphorus being 0 77 to 1 Usually this ratio is closer to 1 for teichoic acids but the relatively low value obtained may be due to the presence of unhydrolysed glycerol phosphates

Free alanine was not detected after incubation of TCA-extracted poly A1 in 1N NH_4OH at 100° C for 10 min

Antigenic Properties

Serum against *S hyicus* VA308 bacteria produced one line against purified poly A1 which was identical to the A1 line produced against a suspension of the homologous bacteria The antiserum did not produce any line against poly C However poly C and poly A1 produced lines of partial identity against antiserum to *S saprophyticus* 3519 the poly C line spurring over that of poly A1 Also against this antiserum the poly A1 preparation produced a weak line identical to the poly C line (Fig 1) The electrophoretic mobility of poly A1 and poly C was nearly the same when tested by immunoelectrophoresis

Double diffusion of purified polysaccharides against Con A showed that both poly A1 and the positive control poly Bα produced lines whereas the negative controls poly C and poly Aβ did not produce any line

Polysaccharides were treated with β N acetylglucosaminidase and subjected to double diffusion Treated poly A1 seemed unaffected and produced lines against both 3519 antiserum and the homologous VA308 antiserum whereas poly C did not give any line against 3519 antiserum The weak poly C line observed in the A1 preparation against 3519 antiserum also disappeared when treated with this enzyme Treatment of the polysaccharides with the N acetylglucosaminidase from *P vulgata* completely destroyed the serological activity of poly A1 *when this* was tested against the homologous VA308 antiserum A weak A1 line against antiserum 3519 however was still observed The serological activity of poly C was also destroyed when treated with this enzyme

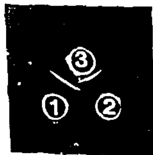


Fig 1 Double diffusion in agar showing a reaction of partial identity between poly C from S *saprophyticus* 3519 (1) and poly A1 from S *hyicus* ss *hyicus* VA 308 (2) against anti 3519 serum (3)

DISCUSSION

The physico-chemical properties of poly A1 appear to be very similar to those of poly C (13 14) Both the binding to DEAE-cellulose and the electrophoretic mobility are nearly the same Also the amounts of phosphorus glycerol and N acetylglucosamine show that poly A1 is a teichoic acid similar to poly C As with poly C poly A1 contains small amounts of attached peptidoglycan This

that poly A1 is a polyglycerol phosphate teichoic acid The only sugar detected was N acetylglucosamine, and when corrected for amino sugar in the peptidoglycan (0 5 μmoles/mg), the relative molar amounts of N-acetylglucosamine to phosphorus is 0 88 to 1 This and the absence of ester-linked D-alanine therefore suggest that poly A1, in contrast to poly C, is almost fully substituted with amino sugar The degree of sugar substitution has previously been found to vary between strains (14)

Con A gave strong precipitation lines with both poly A1 and the positive control poly Bα whereas no line was observed with the negative controls poly Aβ and poly C This indicates that poly A1 contains N-acetylglucosamine in α-configuration (1, 8) In addition the observation that a crude extract, containing α-N-acetylglucosaminidase, in contrast to pure β-N-acetylglucosaminidase destroyed the antigenic reactivity of poly A1 against homologous antiserum supports this The serological activity of poly C was completely lost after treatment with the β-N-acetylglucosaminidase Thus the main difference between poly A1 and poly C seems to be the configuration of the amino sugar Therefore poly A1 ought to be designated poly Cα The finding that the poly Cα preparation from *S hyicus* VA308 also contains small amounts of poly Cβ is consistent with the observations concerning other strains of *S hyicus* (6) It is not unusual that staphylococci contain teichoic acids of two types (α- and β-) (5)

The shared antigenic determinant of poly Cα and poly Cβ seems to be the glycerol phosphate polymer The N-acetylglucosamine has a different configuration in these teichoic acids and can therefore be excluded as the common antigenic determinant Antiserum against poly Cβ has earlier been shown to contain antibodies with specificity against the glycerol phosphate and the amount of these antibodies seems to be dependent on the degree of sugar substitution (14) Therefore poly Cα which is almost fully substituted with N acetylglucosamine may fail to induce antibodies with specificity against the glycerol phosphate This might explain why antiserum VA308 does not cross-react with poly Cβ and may also explain the weak line observed between α N acetylglucosaminidase treated poly Cα and antiserum 3519

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ULTRASTRUCTURAL STUDIES ON THE SPORULATION OF OOCYSTS OF *TOXOPLASMA GONDII*

I Development of the Zygote and Formation of the Sporoblasts

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Ferguson D J P Birch Andersen A Siim J Chr & Hutchison W M Ultrastructural studies on the sporulation of oocysts of *Toxoplasma gondii* I Development of the zygote and formation of the sporoblasts Acta path microbiol scand Sect B 87 171–181 1979

The initial stages of sporulation in oocysts of *Toxoplasma gondii* were examined in samples sporulated at 27°C for 0 6 12 16 and 24 hours The initial zygote was roughly spherical and was limited by a single unit membrane A few micropores of the inactive type were present on this membrane The cytoplasm contained a large nucleus with a nucleolus a number of polysaccharide granules lipid globules mitochondria and Golgi bodies together with a few strands of rough endoplasmic reticulum After the initiation of sporulation little change was noted in the cytoplasm except for an increase in protein synthesis as evidenced by the augmentation of the amount of rough endoplasmic reticulum and the appearance of polyribosomes Nuclear division occurred twice giving rise to four nuclei which were situated close to the cell periphery and well separated from each other At this multinucleate stage a second limiting membrane was formed The cytoplasmic mass then divided to form the two sporoblasts This was accomplished by an invagination of the limiting membranes in combination with internally formed membranes The two binuclear sporoblasts were roughly spherical They were limited by two unit membranes and contained the same cytoplasmic organelles as described for the zygote

Key words *Toxoplasma gondii* sporulation zygote development sporoblast formation ultrastructure

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Toxoplasma gondii is a coccidian parasite of the cat (18) which belongs to the family *Sarcocystidae* (15) When the oocysts of *T gondii* are shed in the cat faeces they are unsporulated but during sporulation two sporocysts are formed each of which contains four sporozoites These disporic tetrazoic oocysts are thus similar to those of the genus *Isospora* which belongs to the family *Eimeriidae*

Ultrastructural studies on the changes which occur during the sporulation of the oocysts of parasites belonging to the families *Eimeriidae* and *Sarcocystidae* have been hampered by difficulties in preparing the oocysts for electron microscopy Recently a technique was developed (1) by which these problems were overcome but to date only the sporulation of oocysts of *Eimeria brunetti* has been examined in detail (9 & 10) Fine structural details of the sporulation of *Isospora* like oocysts have not been reported except for short notes on the sporulation of *T gondii* (7 & 11) In the present paper details of the structural changes observed during the initiation of sporulation of the zygote and the formation of the sporoblasts of *T gondii* will be given and compared with those reported for other members of the Sporozoa

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MATERIALS AND METHODS

Oocysts of *T gondii* were concentrated from the faeces of Specific Pathogen Free cats which had been fed mouse brains containing tissue cysts of either the SSI/119 or the SSI/178 strain of *T gondii*

To study sporulation the oocysts were allowed to sporulate at approximately 27°C for intervals of 0 6 12 16 24 36 and 48 hours before processing for electron microscopy The technique used has been described previously (1) but can be summarised as follows The oocysts were preembedded in cross linked bovine serum albumin (BSA) and frozen in liquid nitrogen prior to cryostat sectioning The cryostat sections were directly immersed in Karnovsky s fixative reembedded in BSA post fixed in osmium tetroxide and finally embedded in either Vestopal of Spurr s epon Sections were stained with magnesium uranyl acetate and lead citrate and examined with a Philips E M 200 electron microscope The results reported are based on the examination of approximately 600 electron micrographs

RESULTS

Light Microscopy

The structure of the oocyst prior to the initiation of sporulation was examined in material processed directly from storage at 4°C Progressive develop mental changes were observed in samples which had been allowed to sporulate for increasing time intervals After 6 hours the oocysts still contained a single cytoplasmic mass (the zygote) but by 12 hours a number of the oocysts had reached the sporoblast stage and a few contained developing sporocysts By 16 and 24 hours the majority of oocysts contained sporoblasts or developing sporo cysts The development is not synchronised and oocysts containing undivided zygotes could still be observed at 24 hours

In toluidine blue stained sections of the 0 hour material the zygote appeared spherical The cyto plasm contained a large centrally located nucleus which was surrounded by a number of polysaccha ride granules and a few lipid globules (Fig 1)

After 6 hours sporulation little change could be seen in the zygote (Fig 2) except that in certain cases the nucleus appeared elongated (Fig 3)

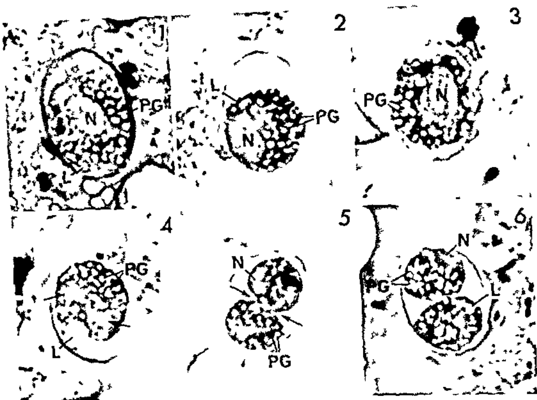
Due to the small size of the oocysts of *T gondii* the changes occurring during the development of the zygote were difficult to ascertain with the light microscope It appeared that nuclear division occurred giving rise to a number of small nuclei but the exact number could not be resolved In general these small nuclei were observed close to the periphery of the zygote (Fig 4)

In sections of 12 and 16 hours sporulated material it was possible to observe oocysts in which the zygote was dividing to form the two sporoblasts At this stage a central constriction which divided the cytoplasm into two equal parts was apparent (Fig 5) The two sporoblasts thus formed were initially spherical and their cytoplasm contained polysaccharide granules and lipid globules The exact number of nuclei present could not be ascertained (Fig 6)

Electron Microscopy

In median sections through unsporulated oocysts (0 hour material) the cytoplasmic mass was seen to contain a large nucleus with amorphous nucleoplasm and a distinct nucleolus The cytoplasm also contained a number of polysaccharide granules a few lipid globules a few Golgi bodies and a number of large mitochondria which in certain cases were situated close to and surrounding the nucleus (Figs 7 & 10) A few strands of rough endoplasmic reticulum were also present (Fig 7) The zygote was limited by a unit membrane which possessed a few inactive micropores (Figs 8 & 9) The outline of the zygote was irregular with a number of small invaginations and evaginations of the limiting membrane (Fig 7)

The developmental changes in the zygote were examined as the oocysts underwent sporulation The overall shape of the cytoplasmic mass remained unchanged (Fig 11) although vacuoles which seemed to be budding off at the periphery were observed in a few organisms (Fig 12) The initial changes in the cytoplasm were minimal but there did appear to be an increase in the amount of rough endoplasmic reticulum (Fig 11) and in the number of ribosomes together with the appearance of polyribosomes In addition a few accumulations of dense material were observed within the cisterna of the rough endoplasmic reticulum (Figs 11 & 13) At this stage certain of the lipid globules were very large reaching 1 7 μm in diameter As the zygote developed the nucleus was displaced to the peri phery of the organism and became elongated (Fig 14) At this stage the nucleus appeared to undergo division During division a nuclear pole with associated centrioles was present at either end of the nucleus (Fig 14) Each centriole consisted of nine microtubules arranged in a circle around a central tubule (Fig 16) The nuclear pole consisted of a dense matrix within which it was difficult to identify the radiating microtubules (Fig 15) The two nuclei formed by the initial division appeared to undergo further division so that a total of four nuclei were present The nuclei thus formed were much smaller in average 2 μm × 1 3 μm in comparison to the original nucleus in the zygote which was approximately 3 4 μm × 2 μm The four small nuclei were situated close to the



Figures 1–6 are light micrographs of 1μm thick toluidine blue *stained* sections and figures 7–24 are electron *micrographs* of thin sections illustrating the development of the zygote and formation of the sporoblasts *within* oocysts of *T gond i*

A double bar (=) on a figure represents 1 μm and a single bar (–) *100 nm*

The *following* abbreviations are used throughout CE – centriole ER rough endoplasmic reticulum G – Golgi body L lipid globule LM – limiting *membrane* MI = mitochondrion MP micropore N nucleus NM nuclear membrane NP – nuclear pole NU – nucleolus OW *oocyst wall* PG polysaccharide granule UM *unit membrane* V vacuole

Fig 1 A section through an unsporulated oocyst (0 *hour*) *The* centrally located nucleus is surrounded by a number of polysaccharide granules and lipid globules In the black and white *prints it* is difficult to differentiate the polysaccharide *granules* from lipid globules but they could be easily identified when examined directly with the light microscope × 2 500

Fig 2 A section through an *oocyst after* 6 hours sporulation The structure is similar to that in Fig 1 except for the peripheral location of the nucleus × 2 500

Fig 3 In this section the nucleus has an elongated appearance × 2 500

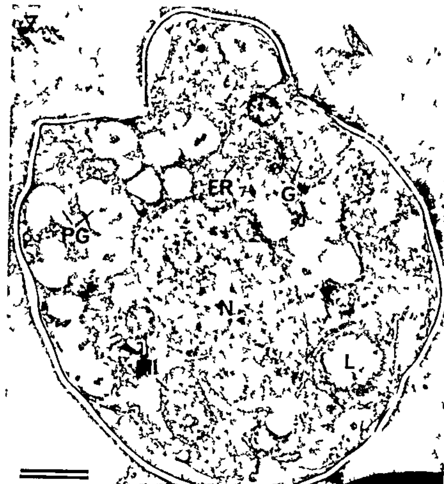
Fig 4 A section through a sporulating oocyst in which the zygote possesses two nuclei (arrows) × 2 500

Fig 5 In this section the cytoplasmic mass has started to *divide to* form the sporoblasts (arrows) × 2 500

Fig 6 A section showing the two spherical sporoblasts × 2 *500*

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periphery of the *organism* and well separated from each other (Fig 17) Each nucleus retained a nuclear pole and associated centrioles which was always located on the side *of the* nucleus towards the periphery of the organism (Figs 17 & 18) During the nuclear di[illegible] *pearance* of the cytoplasmic *organelles* remained unchanged and the cytoplasmic mass was still limited by a *single* unit membrane At the multinucleate stage a second limiting membrane started to develop (Figs 17 & 18) This membrane was *initially* incomplete but finally formed a second unit membrane which


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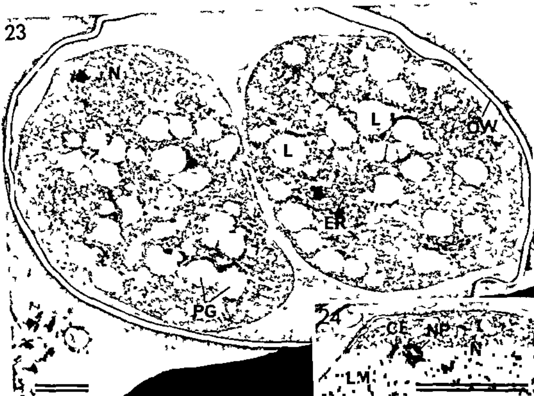


Fig 7 A section through an unsporulated oocyst (0 hour) The cytoplasm contains a nucleus a number of polysaccharide granules lipid globules Golgi bodies mitochondria and a few strands of rough endoplasmic reticulum A nucleolus can be present although not shown in this section × 15 000

Fig 8 An enlargement of part of the periphery of a zygote showing the limiting unit membrane invaginating to form a micropore Note the collar of dense material around the invagination (arrow) × 90 000

Fig 9 A cross section through a micropore of a zygote in which the circular invagination of the limiting membrane and the collar of dense material (arrows) can be seen × 90 000

Fig 10 An enlargement of part of the periphery of the nucleus seen in Fig 7 Note the nuclear membranes and adjacent mitochondrion × 90 000

Fig 11 A section through an oocyst after 6 hours sporulation In addition to the organelles present at the earlier stage the cytoplasm contains accumulations of dense material within some cisterna of the rough endoplasmic reticulum (arrows) × 15 000

Fig 12 In this section of the periphery of a zygote a number of vacuoles appear to be budding off × 30 000

Fig 13 A higher magnification of part of Fig 11 showing the accumulation of dense material (arrow) within the cisterna of the rough endoplasmic reticulum × 30 000

Fig 14 A section through an organism with a dividing nucleus Note the nuclear pole (arrows) with associated centrioles at either end of the elongated nucleus × 30 000

Fig 15 An enlargement of one of the nuclear poles seen in Fig 14 The nuclear pole has a dense matrix within which it is difficult to identify radiating microtubules × 90 000

Fig 16 A detail from a zygote showing a cross cut centriole which consists of nine microtubules (arrows) arranged in a circle round a central microtubule × 90 000

Fig 17 A section through a multi nucleate organism The nuclei are situated close to the periphery of the zygote × 15 000

Fig. 18 A detail of part of Fig. 17. A nuclear pole with associated centriole is observed close to the limiting membrane. Note that a second unit membrane is enclosing part of the zygote. × 90 000

Fig. 19 A section through an oocyst in which the zygote has started to divide (arrow) to form the two sporoblasts. Note the two nuclei present in one of the areas being separated. × 15 000

Fig. 20 A detail of the region where the zygote is dividing. The invagination of the limiting membrane and the presence of unit membranes within the cytoplasm is shown. × 90 000

Fig. 21 A section through part of a zygote in which division into the sporoblasts is almost complete. Note that the invagination of the two limiting membranes appear to combine with the internal unit membranes. × 90 000

Fig. 22 An enlargement of part of the periphery of a sporoblast showing two micropores associated with the inner of the two limiting membranes. × 90 000

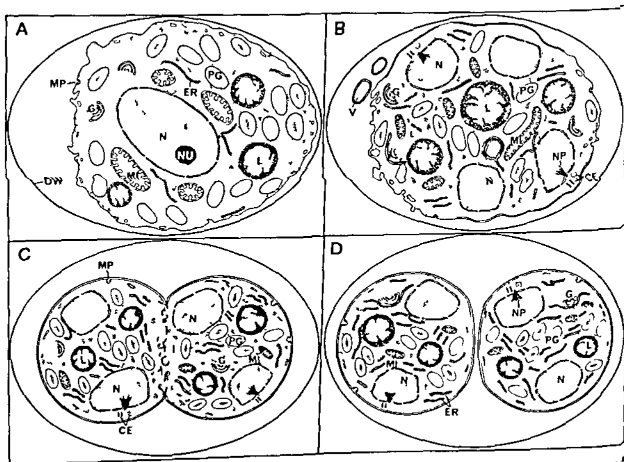
Fig. 23 A section through an oocyst showing the two newly formed sporoblasts. × 15 000

Fig. 24 An enlargement of part of Fig. 23 showing the two limiting membranes of the sporoblast and a nucleus with nuclear pole and associated centriole close to the periphery of the organism. × 30 000

completely enclosed the organism. With the formation of this second limiting membrane the outline of the zygote became smoother in appearance.

After this stage was reached the cytoplasmic mass divided and the two sporoblasts were formed. The division of the cytoplasmic mass initially appeared as a central constriction (Fig. 19). Division apparently was accomplished by invaginations of the limiting membranes which coalesced with internally formed membranes (Figs. 20 & 21). Each of the areas of cytoplasm being separated in this manner contained two nuclei in addition to the cytoplasmic organelles that were present in the zygote (Fig. 19).

The two sporoblasts were roughly spherical in appearance and were limited by two unit membra-



Text Fig. 1 A diagrammatical representation of the changes observed during the development of the zygote and formation of the sporoblasts. A an unsporulated oocyst (0 hour). B a sporulating oocyst. C formation of the sporoblasts. D early sporoblasts.

nes (Figs 20 23 & 24) It was observed that micropores were associated with the inner limiting membrane (Fig 22) The cytoplasm of each sporoblast contained two nuclei plus a number of polysaccharide granules lipid globules Golgi bodies mitochondria and some rough endoplasmic reticulum (Fig 23) A diagrammatical representation of the ultrastructural changes observed during the development of the zygote and the formation of the sporoblasts is given in Text Fig 1

DISCUSSION

In the present study two strains of *T gondii* were examined but no differences in the process of sporulation was observed between the strains As reported for *E brunetti* and *E magna* (9 & 22) the oocysts of *T gondii* when stored at 4°C did not sporulate but retained their viability

The initial sporulation of *T gondii* appeared to be more rapid than that observed for *E brunetti* (9) At 12 hours at 27°C a number of the *T gondii* oocysts had reached the sporoblast stage Thus to observe the initial changes in the zygote an additional sample was processed after 6 hours sporulation

The structure and organelle complement of the zygote is similar to that reported for the developing oocysts of *T gondii* observed prior to their release from the host epithelial cells (14) The zygote of *T gondii* does not exhibit any congregation of the polysaccharide granules as reported for *E brunetti* (9) The *T gondii* zygote however possesses lipid globules which are absent in *E brunetti* (9) The zygote of *T gondii* is limited by a single unit membrane This is similar to that reported for *E tenella* (5) and *E necatrix* (6) and the observation of inactive micropores present on the limiting membrane is similar to that observed for *E brunetti* (8 & 9) A number of large mitochondria are present in the early *T gondii* zygote This observation has not been reported for other species of Sporozoa The ratio of mitochondrial to cytoplasmic volume is large in *T gondii* For this at least two possible explanations can be suggested a) The early zygote has a high energy requirement This seems very unlikely since the oocysts stored at 4°C should be metabolically inactive b) The mitochondria themselves are inactive and for that reason have become swollen This second possibility appears more likely since the mitochondria observed in the later stages of sporulation appear less swollen (cf Figs 7 & 17)

After the initiation of sporulation there was an apparent increase in the protein synthesis as evidenced by augmentation of the amounts of rough endoplasmic reticulum and the appearance of polyribosomes This is similar to that reported for certain other members of the Sporozoa (4 9 & 28) The accumulations of dense material within the cisterna of the rough endoplasmic reticulum may be related to this increase in protein synthesis The development of the zygote was not accompanied by the appearance of dense bodies (precursors of the refractile bodies) as in *E brunetti* (9) nor the crystaloid bodies as in certain other members of the Sporozoa (21 24 & 29) This is probably related to the fact that the sporozoites of *T gondii* (13 & 23) lack both the refractile bodies and the crystaloid bodies which are present in the sporozoites of these other species

The appearance of vacuoles budding off from the surface of the zygote is similar to that reported for *E brunetti* (9) and *Leucocytozoon dubreuili* (29) and as proposed in these studies it is possible that the vacuoles function as a form of waste disposal

The initial zygote possesses a large nucleus probably representing the diploid state which divides twice giving rise to four smaller haploid nuclei This is similar to that reported for *E brunetti* (9) The first division involving the large elongated nucleus probably represents the reduction division but no distinct ultrastructural differences were noted This would be in agreement with the results obtained by microdensitometry of the DNA content during nuclear division in the oocysts of *E tenella* (3) Centrioles and intra nuclear poles were

nuclear

membranes appeared to remain intact during nuclear division This differs from that reported in a light microscope study of *E tenella* (2) In addition we observed no evidence for the presence of multiple nuclear spindles as has been reported in the nucleus of oocysts of *Plasmodium berghei* (4) and schizonts of *Sarcocystis cruzi* & *S suihominis* (19 & 17) Prior to the division of the zygote of *T gondii* into the sporoblasts four nuclei were already formed This sequence of events is similar to that reported for *E brunetti* (9) and *E debliecki* (27)

A second limiting unit membrane was formed around the multi nucleate zygote prior to its division into the sporoblasts Such an extra limiting membrane has not been reported for developing zygotes of any other species of Sporozoa examined to date but the significance of the membrane formation is unclear

The division of the zygote into two sporoblasts appears to be associated with an invagination of limiting membranes in combination with internally formed membranes It thus differs from the straight

Fig. 18. A detail of part of Fig. 17. A nuclear pole with associated centriole is observed close to the limiting membrane. Note that a second unit membrane is enclosing part of the zygote. × 90 000

Fig. 19. A section through an oocyst in which the zygote has started to divide (arrow) to form the two sporoblasts. Note the two nuclei present in one of the areas being separated. × 15 000

Fig. 20. A detail of the region where the zygote is dividing. The invagination of the limiting membrane and the presence of unit membranes within the cytoplasm is shown. × 90 000

Fig. 21. A section through part of a zygote in which division into the sporoblasts is almost complete. Note that the invagination of the two limiting membranes appear to combine with the internal unit membranes. × 90 000

Fig. 22. An enlargement of part of the periphery of a sporoblast showing two micropores associated with the inner of the two limiting membranes. × 90 000

Fig. 23. A section through an oocyst showing the two newly formed sporoblasts. × 15 000

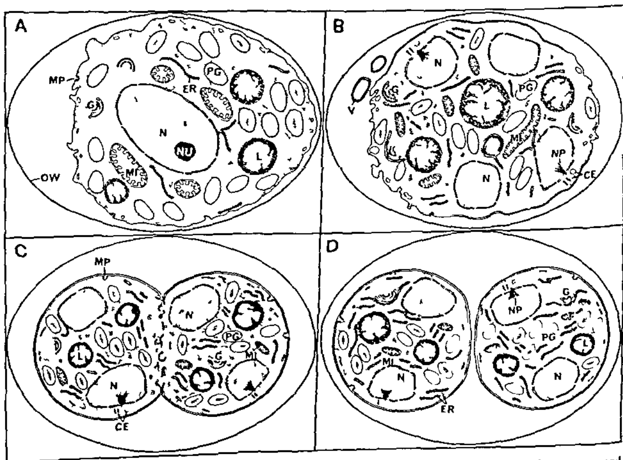
Fig. 24. An enlargement of part of Fig. 23 showing the two limiting membranes of the sporoblast and a nucleus with nuclear pole and associated centriole close to the periphery of the organism. × 30 000

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completely enclosed the organism. With the formation of this second limiting membrane the outline of the zygote became smoother in appearance.

After this stage was *reached*, the *cytoplasmic* mass divided and the two sporoblasts were formed. The division of the cytoplasmic mass initially appeared as a central constriction (Fig. 19). Division apparently was accomplished by invaginations of the limiting membranes which coalesced with internally formed membranes (Figs. 20 & 21). Each of the areas of cytoplasm being separated in this *manner contained* two nuclei in addition to the cytoplasmic organelles that were present in the zygote (Fig. 19).

The two sporoblasts were roughly spherical in appearance and were limited by two unit membra-



Text Fig. 1. A diagrammatical representation of the changes observed during the development of the zygote and formation of the sporoblasts. A, an unsporulated oocyst (0 hour). B, a sporulating oocyst. C, formation of the sporoblasts. D, early sporoblasts.

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forward invagination of the limiting membrane as observed in *E brunetti* and *Hepatozoon domerguei* (9 & 28) but resembles more the coalescence of aligned vesicles as observed in other members of the Sporozoa (20, 21, 25 & 26)

Each of the sporoblasts formed by the zygote in *T gondii* is spherical in shape This is similar to the sporoblasts of *E brunetti* (9) The sporoblasts of these two species are also similar in possessing two limiting membranes although in the case of *E brunetti* the second limiting membrane did not appear until the sporoblasts were formed (9) Micropores were found associated with the inner of the limiting membranes of sporoblasts of *T gondii* This was also the case for sporoblasts of *E brunetti* (8, 9 & 10) and it would appear that the inner membrane represents the plasmalemma while the outer membrane will be concerned with the formation of the sporocyst wall (12)

We are indebted to Mr *J F Dunachie* and Miss *M Reilly* for the maintenance of the SPF cats We gratefully acknowledge Dr *J Blom* for assistance with the light microscopy Mrs *H Raun* and Mrs *J Berg* for technical assistance and Miss *A G Overgaard* and Mr *F Laursen* for photographic assistance

The work was supported by grants from the *World Health Organisation* Geneva the *Wellcome Trust* the *Danish Medical Research Council* and the *W H Ross Foundation*

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ULTRASTRUCTURAL STUDIES ON THE SPORULATION OF OOCYSTS OF *TOXOPLASMA GONDII*

II Formation of the Sporocyst and Structure of the Sporocyst Wall

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FAO/WHO Collaborating Centre for Research and Reference in Toxoplasmosis[1] and Department of Biophysics[2] Statens Seruminstitut Copenhagen Denmark and Department of Biology[3] University of Strathclyde Glasgow Scotland

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The ultrastructural changes observed during sporocyst formation and the structure of the sporocyst wall was examined in oocysts which had been allowed to sporulate for between 12 and 48 hours at 27°C As the spherical sporoblast developed into the sporocyst the cytoplasmic mass became ellipsoidal in shape although no change was noted in the organelle compliment which consisted of two nuclei plus a number of polysaccharide granules lipid globules mitochondria Golgi bodies and some rough endoplasmic reticulum The sporocyst wall consisted of a thin outer layer (15–20 nm) which was formed from two limiting membranes of the sporoblast and an inner layer (40–50 nm) which was comprised of four curved plates This inner layer was formed under the outer layer and although no specific cytoplasmic organelle disappeared with its formation some unit membranes were observed close to the plasmalemma during its formation Each curved plate has a marginal swelling and an interposing strip of material is present between the margins of adjacent plates The plates are joined to the interposing strip by a thin band of osmiophilic material In oblique and tangential sections through the plates two types of cross banding were observed which differed in periodicity

Key words *Toxoplasma gondii* oocysts sporocyst sporocyst wall ultrastructure

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Received 18 ix 78 Accepted 13 xii 78

The sporulated oocysts of *Toxoplasma gondii* possess two sporocysts each of which contain four sporozoites and are thus similar to the genus *Isospora* Due to difficulties in preparing these *Isospora* like oocysts for electron microscopy the subcellular changes accompanying sporocyst wall formation have not been described although the ultrastructure of the sporocyst wall after either physical disruption or treatment with excysting enzymes has been reported for *Isospora canis* and *I endocallimici* (15 16) In this paper using a recently developed technique (1) details will be given on the formation of the sporocyst and the ultrastructure of the fully developed sporocyst wall of *T gondii*

Figures 1–18 are electron micrographs of sections illustrating the formation of the sporocyst and structure of the sporocyst wall within the oocysts of *T gondii*

A double bar (=) on a figure represents 1 µm and a single bar (–) 100 nm

The following abbreviations are used throughout ER = rough endoplasmic reticulum I inner layer of the sporocyst wall IS = interposing strip L = lipid globules LM = limiting membranes MI = mitochondrion N = nucleus O = outer layer of the sporocyst wall P – plasmalemma PG = polysaccharide granule UM unit membrane

Fig 1 A section through a spherical sporoblast The cytoplasm contains two nuclei and a number of polysaccharide granules lipid globules mitochondria and a few strands of rough endoplasmic reticulum × 15 000

Fig 2 In this section through a sporoblast at a slightly later stage of development than that in Fig 1 the organism has attained an ellipsoidal appearance × 15 000

Fig 3 An enlargement of part of the periphery of the organism in Fig 1 showing the sporoblast to be limited by two unit membranes × 90 000

Fig 4 An enlargement of part of the periphery of the organism in Fig 2 At this stage the organism is limited by three unit membranes × 90 000

Fig 5 Part of the periphery of an organism which shows the early stage of formation of the inner layer of the sporocyst wall (arrows) × 160 000

Fig 6 A section through the periphery of a forming sporocyst At this stage fine striations can be seen in the developing inner layer of the sporocyst wall (arrows) × 90 000

Fig 7 In this section through the periphery of a forming sporocyst the anlage of the junction between two of the developing plates which form the inner layer of the sporocyst wall can be seen (arrow) × 90 000

Fig 8 A section showing the anlage of the junction between two of the developing plates of the inner layer of the sporocyst wall (arrow) Note the unit membranes adjacent to the plasmalemma × 160 000

Fig 9 An enlargement of part of a fully formed sporocyst wall Note that the two membranes of the outer layer have lost their unit membrane appearance × 90 000

MATERIALS AND METHODS

The materials and methods were as described previously (1 4) In this study oocysts which had been allowed to sporulate for 12 16 24 36 and 48 hours were examined The results are based on the examination of approximately 600 electron micrographs

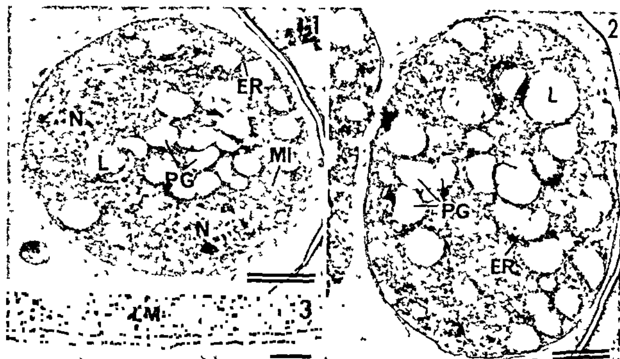
RESULTS

The n 1

... the two sporoblasts are formed by division of the zygote Each sporoblast is initially sperical in shape and limited by two unit membranes (Figs 1 3) but an additional unit membrane soon appears (Fig 4) The two outer membranes will eventually become the outer layer of the sporocyst wall while the inner membrane represents the plasmalemma of the cytoplasmic mass At this stage the sporoblast starts to become ellipsoidal in shape (Fig 2)

The first evidence of the formation of the inner layer of the sporocyst wall is the accumulation of a thin layer of material between the plasmalemma and the second limiting membrane (Fig 5) As this layer continues to increase in thickness a cross striated substructure with a periodicity of 3 5 nm becomes visible (Figs 6 7) As the inner layer forms swellings can be observed which represent the anlagen of the specialised junctions of the plates

... of two nuclei plus a number of polysaccharide granules lipid globules mitochondria Golgi bodies and some rough endoplasmic reticulum (cf Figs 1 10) No specific cytoplasmic organelle disappeared with formation of the sporocyst wall although during its formation some unit membranes were observed close to the plasmalemma (Fig 8)


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F g 5 Part of the periphery of an organism which shows the early stage of formation of the inner layer of the sporocyst wall (arrows) × 160 000

F g 6 A section through the periphery of a forming sporocyst At this stage fine striations can be seen in the developing inner layer of the sporocyst wall (arrows) × 90 000

F g 7 In this sect on through the periphery of a forming sporocyst the anlage of the junction between two of the develop ng plates which form the inner layer of the sporocyst wall can be seen (arrow) × 90 000

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RESULTS

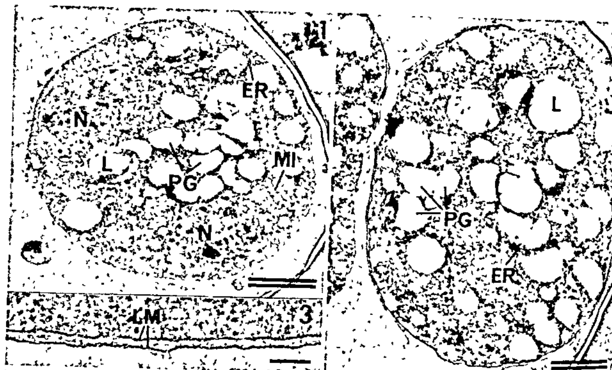
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Sporocyst wall formation is initiated soon after the two sporoblasts are formed by division of the zygote Each sporoblast is initially sperical in shape and limited by two unit membranes (Figs 1 3) but an additional unit membrane soon appears (Fig 4) The two outer membranes will eventually become the outer layer of the sporocyst wall while the inner membrane represents the plasmalemma of the cytoplasmic mass At this stage the sporoblast starts to become ellipsoidal in shape (Fig 2)

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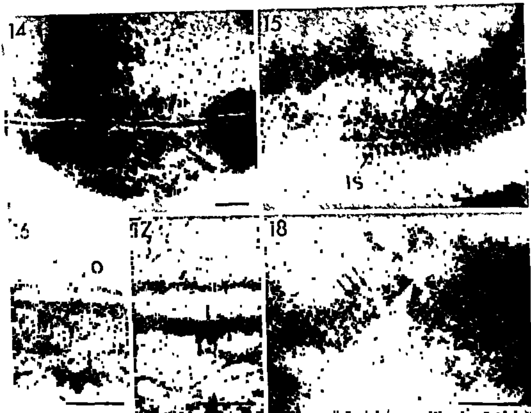


Fig 14 A tangential section through the inner layer of the sporocyst wall showing the banded appearance of the interposing strip when cut longitudinally × 90 000

Fig 15 A tangential section through the inner layer of the sporocyst wall The banded appearance of the interposing strip and the coarse striation on one of the plates (arrows) is evident × 160 000

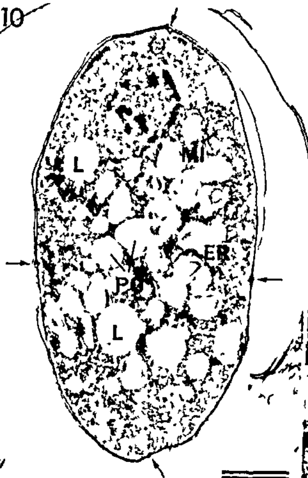
Fig 16 An oblique section through the sporocyst wall showing the coarse striations in the inner layer (arrows) × 160 000

Fig 17 A tangential section through the inner layer of the sporocyst wall In this case fine striations can be seen (arrows) × 160 000

Fig 18 In this section the inner layer of the sporocyst wall is cut tangentially and the two types of striations can be seen superimposed on each other The coarse striations (arrow heads) are at an angle of approximately 70° to the fine striations (arrows) × 160 000

distinct osmiophilic terminal band (Figs 12 13) In tangential sections however some fine striations are visible on the surface of the inner layer (Figs 17 18) and in oblique sections through this layer a second coarse type of striation can be seen in which the electron-dense and lucent bands are 3 5 nm and 10 nm wide respectively (Figs 15 16) In a few cases the two types of striations were observed superimposed on each other and it was observed that the fine striations were at an angle of approximately 70° to the coarse striations (Fig 18)

The margin of each plate is swollen to a thickness of approximately 100 nm The plates are joined by a complex structure in which an interposing strip of material is situated midway between the swollen margins of the adjacent plates (Figs 12 13) This strip is 15 nm thick and 130 nm wide and presents two osmiophilic edges (Fig 13) In longitudinal sections through the strip it also displays a cross banding (Figs 14 15) A space of approximately 5 nm is observed between the strip and the margins of the two adjoining plates and the plates are connected



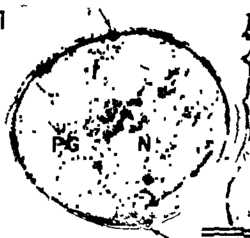






Fig 10 A longitudinal section through a sporocyst The junctions of the four plates of the inner layer of the sporocy wall can be seen (arrows) The junction at the bottom is not too evident in this section but its presence has bee confirmed in serial sections × 15 000

Fig 11 A transverse section through a sporocyst The two junctions between the plates present in the plane of th section are shown (arrows) × 15 000

Fig 12 A cross section through the sporocyst wall in the region of the junction of two plates The interposing strip and the thin bands of osmiophilic material (arrows) joining the plates to the interposing strip can be seen Note the depression in the outer layer (arrow head) directly over the junction × 90 000

Fig 13 A similar section to that shown in Fig 12 The thin bands of osmiophilic material joining the plates to the interposing strip (arrows) are seen × 90 000

The fully formed sporocyst wall is thus comprised of two layers The outer layer consists of the two membranes previously mentioned which at this stage have lost their distinct unit membrane structure and are normally found closely applied to each other (Figs 12 13) In addition some amorphous material may be present on the outer surface or this layer The inner layer is comprised of four curved plates although one example of a sporocyst possessing five plates was observed in excystation studies (unpublished results) The plates have a fixed orientation within the sporocyst wall This configuration is diagrammatically represented in Text Fig 1 From this it would appear that in longitudinal sections through the sporocyst the joins of all four plates are visible (Fig 10) while in transverse sections joins of only two plates can be seen (Fig 11) Normally cross sections through the fully formed plates have a homogenous appearance although in certain cases cross striations are apparent These striations ved close to the margins of

endocall n ci (15 16) and also to other members of the coccid a e g *A eberth* (11 12) *C d rchoni* (8 9) and *Del or i a stl e ela s* (10) In *T g id i* we observed a shallow depress on in the outer layer of the sporocyst wall directly over the jo n ng region of the plates of the inner layer This observation has apparently not been made in the other species stud ed The inner layer of the sporocyst wall of *T gond* consists of four plates (the s ngle observation of a sporocyst w th five plates was except onal and atyp cal) Th s is similar to the wall structure demonstrated in sporocysts of *I ca s* and *I e docall ci* (15 16) and the orientat on of the plates would appear to be identical to that reported for *I e docall n ci* (16) In *I e docall i ci* the cross striat on in the sporocyst wall appears to be l m ted to the marginal reg ons of the four plates (16) In *T go d* we have observed two distinct types of striat ons One type with fine striat ons was observed n tangent al sections of the surface of the plates and the other type w th coarser striat ons was observed n obl que sections through the plates Two d st nct types of str ations have also been reported n tangential sect ons of the inner layer of the sporocyst wall of *A ebertl C d rcl o* and *D s l e ela s* (12 9 10) The period cities of these appear to be similar to the f ne and coarse striat ons observed n *T gond* The observat ons on *T gond i* are sim lar to those on the other species in that when the two types of str ations were observed in the same plane of the sections they were at an angle to each other

The plates of the nner layer of the sporocyst wall are joined by a complex structure which has been termed the dch scence dev ce n *A cbertl* (11 12) *C d rcl o* (8 9) and *D stl e ela s* (10) In *T gond* the substructure of these joints with a

appears to be present in *A ebertl* (11 12) *C durcl on* (8 9) and *D stl enela s* (10) and the banded appearance of th s str p in long tud nal sections in *T g nd i* is s m lar to that reported for *A ebertl i* and *D stl e ela s* (11 10) In *T go d* the plates are jo ned to the interposing str p by a th n band of osm oph l c mater al This conf guration is d fferent from that reported for *I endocall m c* where a thin layer of material above the nterposing strip jo ned the adjacent plates directly (16) In *T go d* the presence of a 5 nm space between the plates and the nterposing strip d ffers from that reported for *I endocall n ci* where no space was observed (16)

From our study it would appear that the structure of the sporocyst wall of *T gond* is sim lar to that of the closely related species *I can s* and *I endocall m ci* In addition it possesses many of the characteristics reported for the sporocyst walls of *A eberthi C durcl i* and *D stl cnela s* On the other hand the structure of the sporocyst wall of all these species is basically d fferent from that reported for other members of the genus *Isospora* (e g *I scr* (14)) and the genus *E mcr a* (2 13) In these the sporocyst wall has a single aperture contain ng a Stieda body with an associated sub Stieda body The signif cance of this bas c d fference in the structure of the sporocyst wall between these closely related species and the poss ble evolutionary process by which it has become established is diff cult to explain

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We a e indeb ed to Mr *J F D a l* and Miss *M Re ll* for the ma ntenance of the SPF cats We gra efully acknowledge Mrs *H Ra* and Mrs *J B rg* for techn cal ass tance and M s *A C O rgaard* and Mr *F Lau sen* fo pho ograph ass tance

The work wa suppor ed by grants from the *W ld Heal l Organ a* Geneva the *W ll Tr s* the *Da sl M d al R ar l C l* and the *W H R s Fo nda o*

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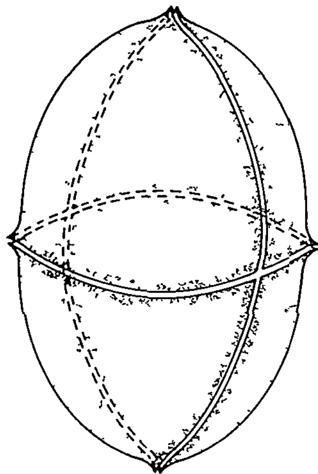
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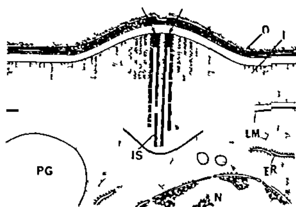
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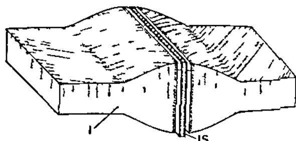
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der Makrogame en und der Sporen Z Paras enk *33* 44–64 1969



Text Fig 1 A three dimensional representation of the inner layer of the sporocyst wall showing the arrangement of the four curved plates



Text Fig 2 A diagrammatical representation of a cross section through the sporocyst wall in the region of a junction between two of the plates of the inner layer The plates are joined to the interposing strip by a thin band of osmiophilic material (arrows)



Text Fig 3 A three dimensional representation of the inner layer of the sporocyst wall at the junction between two plates

to the strip and thus to each other by a thin band of *osmiophilic material situated towards the exterior of* the margins and the interposing strip (Figs 12 13) Finally it should be noted that a shallow depression is observed in the outer layer of the sporocyst wall at a position directly over the joins of the plates (Fig 12) Diagrammatical representations of the three dimensional appearance of the joining region of the plates (Text Fig 3) and the cross sectional appearance of the complete sporocyst wall (Text Fig 2) are given

DISCUSSION

The formation of the sporocyst wall has not been described for *Isospora* like oocysts However the present study has shown that in *T gondii* the outer layer is formed from the limiting membranes of the sporoblast This condition is similar to that described for the related coccidians *Aggregata eberthi* (12) and *Coelotropha durchoni* (8 9) In *T gondii* the inner layer of the sporocyst wall is similar in its resistance to the penetration of chemical reagents as that of the oocyst wall (5) In oocyst wall formation two types of wall forming bodies were concerned with and disappeared during its formation (5) However the formation of the sporocyst wall was not accompanied by the disappearance of any specific cytoplasmic organelle This is similar to that reported for *C durchoni* (8 9) and *E brunetti* (2) but differs from the reports on *A eberthi* (6) and *Sarcocystis tenella* (7) where granular bodies and osmiophilic bodies were *thought to be involved in the formation of the* sporocyst wall In the majority of organisms the formation of the sporocyst wall precedes the initiation of sporozoite formation (3) This differs from that reported for *E brunetti* (2)

Our material shows that the sporocyst wall of *T gondii* is made up of a thin outer layer and a thicker inner layer consisting of a number of curved plates This basic structure is similar to that reported for the two closely related species of *I* can s and *I*

endocallimici (15 16) and also to other members of the coccidia e g *A eberthi* (11 12) *C durchoni* (8 9) and *Dehornia sthenelais* (10) In *T gondii* we observed a shallow depression in the outer layer of the sporocyst wall directly over the joining region of the plates of the inner layer This observation has apparently not been made in the other species studied The inner layer of the sporocyst wall of *T gondii* consists of four plates (the single observation of a sporocyst with five plates was exceptional and atypical) This is similar to the wall structure demonstrated in sporocysts of *I canis* and *I endocallimici* (15 16) and the orientation of the plates would appear to be identical to that reported for *I endocallimici* (16) In *I endocallimici* the cross striation in the sporocyst wall appears to be limited to the marginal regions of the four plates (16) In *T gondii* we have observed two distinct types of striations One type with fine striations was observed in tangential sections of the surface of the plates and the other type with coarser striations was observed in oblique sections through the plates Two distinct types of striations have also been reported in tangential sections of the inner layer of the sporocyst wall of *A eberthi* *C durchoni* and *D sthenelais* (12 9 10) The periodicities of these appear to be similar to the fine and coarse striations observed in *T gondii* The observations on *T gondii* are similar to those on the other species in that when the two types of striations were observed in the same plane of the sections they were at an angle to each other

The plates of the inner layer of the sporocyst wall are joined by a complex structure which has been termed the »dehiscence device« in *A eberthi* (11 12) *C durchoni* (8 9) and *D sthenelais* (10) In *T gondii* the substructure of these joints with a marginal swelling of the plates and an interposing strip is similar to that reported for *I canis* and *I endocallimici* (15 16) The interposing strip also appears to be present in *A eberthi* (11 12) *C durchoni* (8 9) and *D sthenelais* (10) and the banded appearance of this strip in longitudinal sections in *T gondii* is similar to that reported for *A eberthi* and *D sthenelais* (11 10) In *T gondii* the plates are joined to the interposing strip by a thin band of osmiophilic material This configuration is different from th

[illegible] of a 5 nm space between the plates and the interposing strip differs from that reported for *I endocallimici* where no space was observed (16)

From our study it would appear that the structure of the sporocyst wall of *T gondii* is similar to that of the closely related species *I canis* and *I endocallimici* In addition it possesses many of the characteristics reported for the sporocyst walls of *A eberthi* *C durchoni* and *D sthenelais* On the other hand the structure of the sporocyst wall of all these species is basically different from that reported for other members of the genus *Isospora* (e g *I serini* (14)) and the genus *Eimeria* (2 13) In these the sporocyst wall has a single aperture containing a Stieda body with an associated sub Stieda body The significance of this basic difference in the structure of the sporocyst wall between these closely related species and the possible evolutionary process by which it has become established is difficult to explain

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We are indebted to Mr J F Dunachie and Miss *M Reilly for the maintenance of the SPF cats* We gratefully acknowledge Mrs *H Ravn* and Mrs *J Berg* for technical assistance and Miss *A G Overgaard* and Mr *F* [illegible]

H[illegible]

Danish Medical Research Council and the *W H Ross Foundation*

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Acta path microbiol scand Sect B 87 191–195 1979

QUANTIFICATION OF TETANUS ANTITOXIN IN HUMAN SERA

I Counter-Immunoelectrophoresis

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Winsnes R Quantification of tetanus antitoxin in human sera I Counter immunoelectrophoresis Acta path microbiol scand Sect B 87 191–195 1979

An extensive sero epidemiological survey of immunity to tetanus is to be performed in Norway during 1978 and 1979 and thus a simple and reliable method for screening sera for content of tetanus antitoxin is needed An improved counter immunoelectrophoretic method for quantification of tetanus antitoxin is described The toxin neutralization test in mice is considered to correlate well with protection in humans Counter immunoelectrophoresis has the advantage of using tetanus toxoid instead of toxin as well as being more rapid and less expensive Specific antibodies of the IgG IgM and IgA classes could be read simultaneously for many sera This may be of importance since it is reported in the literature that only antibody of the IgG class is capable of neutralizing tetanus toxin in mice Counter immunoelectrophoresis is limited by being less sensitive than the toxin neutralization test in mice

Key words Counter immunoelectrophoresis tetanus antitoxin

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Administration of diphtheria pertussis tetanus vaccine has resulted in an almost total eradication of tetanus in Norway During the period 1963–1977 only ten deaths due to tetanus were reported Since a correlation between antitoxin level and untoward reactions seems to exist (9 20 24) the immunization schedules will be re-evaluated taking into account the antitoxin level necessary for protection

As the correlation between passive haemagglutination (4 22) ...

phoretic method has been investigated

As far as is known no thorough study of the counter immunoelectrophoretic method for determination of tetanus antitoxin has been published hitherto A brief report dealing with quantification of tetanus antitoxin by counter immunoelectrophoresis has been published (21) The experimental conditions described in that study were found to be unsuitable in our hands A modification (6) of the method of *Sgouris* (1972) has been mentioned briefly in the literature The improved method described in the present study was found to be suitable in studies of immune status after diphtheria pertussis tetanus vaccination (26)

MATERIALS AND METHODS

Counter immunoelectrophoresis Glass plates (9 2 × 8 4 cm) were coated with 0 1 cm layer of 1 per cent agarose (Agarose A 37 from L'Industrie Biologique Francaise) ...

... into the gel A slide of this size accommodated three double rows with nine paired wells in each row Thus 27 specimens could be analysed simultaneously on one slide Serum samples (10 µl/well) were placed in the rows of wells near the anode

Electrophoresis was carried out for 60 min before tetanus toxoid (10 µl/well) was added to the rows nearest to the cathode after which electrophoresis was continued for a further 30 min

Electrophoresis was run at 6 V/cm using a water cooled apparatus and the barbital buffer described above The agar plates were allowed to stand in a moist chamber overnight before reading Dilutions of a high titred tetanus immunoglobulin preparation (Statens Bakteriolo giska Laboratorium (SBL) Stockholm) were used as controls

Two different preparations of tetanus toxoid were used as antigens The least purified toxoid was precipitated by TCA according to standard procedures and after dilution in 0 9 per cent saline contained 5 5 Lf/ml and 5 4 µg protein nitrogen/ml determined by a micro Kjeldahl technique after TCA precipitation and heat coagulation (18) (Lf = Limes flocculationis the amount of toxin or toxoid which when mixed with one International Unit of antitoxin gives a Ramon floccula tion in the shortest time International Unit (I U) of antitoxin is the specific activity of a stated amount of the International Standard as defined by the WHO Expert Committee on Biological Standardization (2))

The other tetanus toxoid preparation was purified further by gel filtration on a column (75 × 1 5 cm) of Bio Gel A 1 5 m (Bio Rad Laboratories USA) equilibrated and eluted with 0 1 M tris HCl buffer pH 8 0 in 0 5 M NaCl Concentration was performed in Minicon A 75 (Amicon Corporation USA) concentra tors for macrosolutes The concentrate was dialysed against 0 9 per cent saline and diluted to contain 5 5 Lf/ml of toxoid

The toxoid antigen preparations were analysed by polyacrylamide slab gel electrophoresis in the presence of sodium dodecyl sulphate using a discontinuous buffer system (7) based on the method of *Laemmli* (14) Human albumin (mol wt 68 000) and ovalbumin (mol wt 43 000) were used as references

The toxoids (3 Lf/10 µl) were also analysed by crossed immunoelectrophoresis against unpurified teta nus antitoxin of equine origin against purified human tetanus immunoglobulin (SBL) of the IgG class and against one of the human sera forming two precipitation lines between the wells in counter immunoelectrophore sis Crossed immunoelectrophoresis was performed according to *Weeke* (1973) using 1 per cent agarose (Agarose A 37) in a barbital glycine/tris buffer pH 8 8 ionic strength 0 08 For the first run 2 8 Lf/10 µl of tetanus toxoid was added The field strength was 10 V/cm and the electrophoresis time was 1 h in the first dimension For the second run tetanus antitoxin (9 6 I U/ml agarose solution) was added In the second dimension a field strength of 2 5 V/cm and an electrophoresis time of 16 h were applied

Identification of immunoglobulin classes participating in the precipitation lines Some sera formed two lines between the wells in counter immunoelectrophoresis In order to determine the immunoglobulin class participa ting in the precipitation lines the sera were fractionated IgM was separated from IgG and IgA by gel chromato graphy on a column similar to the one used for purification of tetanus toxoid The IgM fraction collected from the column was identified by immuno double diffusion in 1 per cent agarose gel (9 mm from centre to centre of wells) using antihuman IgM obtained from Behringwerke Germany The IgM and the IgG fractions were tested by immuno double diffusion against antihuman IgG and antihuman IgA from the same commercial source The IgM fraction was further purified from IgG aggregates by immuno adsorption on a column (0 9 × 7 0 cm) of anti pFc coupled to agarose Anti pFc is specific for IgG as pFc is the C terminal domain in the IgG molecule The immunosorbent was a gift from Dr *Terje Michaelsen* Oslo IgM was eluted with phosphate buffered saline pH 7 2 The purified IgM fraction was tested by counter immunoelectropho resis against tetanus toxoid

The fraction containing non aggregated IgG was purified from IgA by ion exchange chromatography on a column (30 × 1 5 cm) of Whatman DE52 cellulose (W &R Balston Ltd England) IgG was eluted with 0 015 M phosphate buffer pH 7 6

Some sera formed a precipitation line located anodically to the serum well In order to identify the antibody class participating in this line a third well was filled with 10 µl of fluoresceinisothiocyanate-conjugated antihuman IgA antibodies (8 mg/ml) the day after electrophoresis The molar ratio of fluoresceinisothiocy anate to IgA was 1 5 Alternatively the third well was filled with 10 µl of fluoresceinisothiocyanate conjugated antihuman IgG (5 mg/ml) The molar ratio of fluoresce insothiocyanate to IgG was 3 3 The conjugates were allowed to diffuse in the agarose gel for 48 h in a humid chamber before the plates were washed in phosphate buffered saline pH 7 2 When these gel plates were photographed a combination of short waved UV light through the gel and weak daylight on the gel was used (Kodak TRI X Pan Film ASA 400)

The fluoresceinisothiocyanate conjugates were the gifts of Dr *Terje Michaelsen*

Sera The human serum specimens used in the preparation of the counter immunoelectrophoretic meth od were taken from persons vaccinated within the previous three months Sera found to be negative were retested after being concentrated by Minicon S 125 or by lyophilization

RESULTS

Standardization of the counter immunoelectrophore tic assay The least purified tetanus toxoid prepara tion used as antigen in counter immunoelectropho resis contained 1022 Lf/mg protein nitrogen The sensitivity for detection of antitoxin was highest at a toxoid concentration of 5 5 Lf/ml At higher antigen dilutions the precipitate became weak

The other tetanus toxoid preparation used as antigen contained 3520 Lf/mg protein nitrogen The optimal antigen prepar

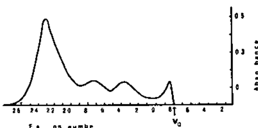


Fig 1 Gel filtration of tetanus toxoid containing 1022 Lf/mg protein nitrogen on Bio Gel A 1 5 Absorbance at 278 nm (—) and at 254 nm (--) of the eluate was determined

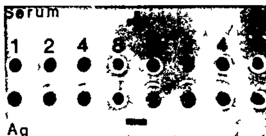


Fig 3 Counter immunoelectrophoresis of two serum samples against tetanus toxoid containing 3520 Lf/mg protein nitrogen Twofold serum dilutions were used

ml Tetanus toxoid was eluted from the Bio-Gel column in fractions 11–14 (Fig 1) The ratio $A_{280\,nm}/A_{254\,nm}$ for the toxoid peak varied between 1 4 and 1 6 in different experiments The purification degree achieved is illustrated by polyacrylamide slab gel electrophoresis in Fig 2 The toxoid containing 3520 Lf/mg protein nitrogen formed a single band staining for protein while the preparation containing 1022 Lf/mg protein nitrogen formed eight additional bands

The same arithmetic mean titres were obtained for the serum specimens concerned by the two toxoid preparations in counter immunoelectrophoresis In repeated tests the titres could vary by one titre step using twofold dilutions The same degree of variation was seen in the toxin neutralization tests

Most of the sera formed one distinct precipitation line in counter immunoelectrophoresis As shown in Fig 3 the precipitate at the dilution taken as end point was easily distinguishable from the negative reaction of the succeeding serum dilution With both toxoids some sera with a relatively high content of tetanus antitoxin formed a well-defined additional line between the wells The serum forming two precipitation lines in Fig 3 was not titrated to negative reaction The figure illustrates that the additional precipitation line is not visible at higher serum dilutions The IgG fraction of a serum forming two precipitation lines between the wells purified by gel filtration and ion exchange chromatography was responsible for the major line Reading of titres was based on this major precipitation line in which IgG antibodies participate The IgM fraction containing IgG aggregates also formed a line in counter immunoelectrophoresis When the IgG aggregates were removed by immuno-adsorption the IgM fraction still gave a precipitation line The presence of IgM and absence of IgG were proved by immuno double diffusion against anti IgM and anti IgG after concentration of the eluate from the immunosorbent column to the original volume followed by agarose gel electrophoresis

By crossed immunoelectrophoresis both tetanus toxoid antigens formed one precipitation line with human tetanus immunoglobulin of the IgG class whereas both toxoid preparations formed two

[illegible] However the precipitation lines were more distinct for the most purified toxoid preparation (Fig 4)

A few sera formed a precipitation line located anodically to the serum well This line appeared both in the presence and absence of the IgG precipitation line (Fig 5) Addition of fluorescein

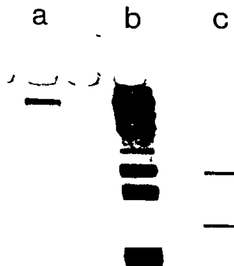


Fig 2 Polyacrylamide slab gel electrophoresis of tetanus toxoid containing 3520 Lf/mg protein nitrogen (a) tetanus toxoid containing 1022 Lf/mg protein nitrogen (b) and a mixture of human albumin and ovalbumin (c)

Fig 4 Crossed immunoelectrophoresis against equine anti tetanus serum of tetanus toxoid containing 3520 Lf/ mg protein nitrogen Anode to the right and top

isothiocyanate conjugated anti IgA made the anodically located line fluorescent and more distinct As illustrated in Fig 5 conjugated anti IgG had no such effect This indicated that anti tetanus antibodies of the IgA class were present in the precipitate

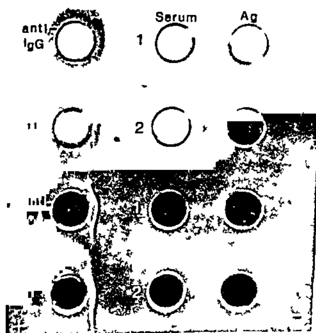


Fig 5 Counter immunoelectrophoresis of two serum samples (No 1 & 2) against tetanus toxo d containing 3520 Lf/mg protein nitrogen Fluoresceinisothiocyanate conjugated anti human IgG or conjugated anti human IgA was added to the additional set of anodically located wells the day after electrophoresis

DISCUSSION

Counter immunoelectrophoresis has often been used for identification of antigen (3 5 8) or antibody (1 19) The present results as well as a report by others (16) indicate that counter immunoelectrophoresis may be employed for quantification of human antibodies The sensitivity of the method could be improved by concentration of sera As discussed by others (13 19 23) it is of importance to run carefully calibrated concentration of antigens and the sera in several dilutions

Many sera especially the high titred ones formed an additional precipitation line between the antigen and the serum well As the IgM serum fraction tested formed a precipitation line against highly purified tetanus toxoid the additional line was probably due to IgM antibodies Furthermore it was unlikely that the highly purified tetanus toxoid giving one band staining for protein on slab gel electrophoresis should contain two different antigens Not only the size but also the electrophoretic mobility in that case would have to be the same according to the results of crossed immunoelectrophoresis Consistent with this conclusion the human tetanus immunoglobulin preparation containing immunoglobulin of the IgG class gave a single precipitation line by crossed immunoelectrophoresis

Hernandez et al (1973) reported that the appearance of transient tetanus antibody of the IgM and IgA class is not limited to the primary immune response

Ourth & MacDonald (1977) found that IgG was the human immunoglobulin class of importance in neutralizing tetanus toxin in mice The low neutralizing capacity of IgM and IgA could be due to IgG contamination Furthermore adsorption of rabbit antisera to tetanus toxoid with goat antirabbit Fc specific for adsorption of IgG from antiserum gave antibodies incapable of neutralizing tetanus toxin Taking the above mentioned reports into consideration it must be of the utmost importance to supplement the toxin neutralization test in mice with a rapid and inexpensive method which assays anti tetanus antibodies of the IgG class only The counter immunoelectrophoretic method will be evaluated further for possible routine use (26)

It was proposed recently to replace or to complete the *in vivo* titration of tetanus antitoxin by radial immunodiffusion (15) However radial immunodiffusion may give too high antibody titres of many sera as precipitins of the IgM and the IgA class will be included

As recently proposed by *Hardegree et al* (1978) anti tetanus antibodies of the IgE class should also

be quantified if possible in the evaluation of hypersensitivity reactions following immunization

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The skilled technical assistance of *Helen Bergsvik* is gratefully acknowledged

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Acta path microbiol scand Sect B 87 197–200 1979

QUANTIFICATION OF TETANUS ANTITOXIN IN HUMAN SERA

II Comparison of Counter Immunoelectrophoresis and Passive Haemagglutination with Toxin Neutralization in Mice

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Winsnes R & Christiansen G Quantification of tetanus antitoxin in human sera II Comparison of counter immunoelectrophoresis and passive haemagglutination with toxin neutralization in mice Acta path microbiol scand Sect B 87 197 200 1979

We described recently an improved counter immunoelectrophoretic method for quantification of tetanus antitoxin The toxin neutralization test in mice is considered to correlate well with protection in humans In the present study the correlation coefficient between the two methods was 0 89 Sera containing more than 7 0 I U /ml could be quantified directly by counter immunoelectrophoresis while sera containing less tetanus antitoxin had to be concentrated prior to quantification The passive haemagglutination test was also compared with the toxin neutralization test in mice The correlation coefficient between the two methods was 0 76

Key words Counter immunoelectrophoresis haemagglutination tests neutralization tests tetanus antitoxin

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The toxin neutralization test in mice is demanding from the point of view of animal facilities and causes suffering to the animals It is also time consuming since it takes five days to analyse a serum sample In order to supplement *in vivo* titration by a more rapid and simple method the possible usefulness of counter immunoelectrophoresis for quantification of tetanus antitoxin was recently investigated (9) Specific antibodies of the IgG IgM and IgA classes could be read simultaneously for many sera Since only antibodies of the IgG class seem to be capable of neutralizing tetanus toxin in mice (6) counter immunoelectrophoresis may be a suitable and rapid method for demonstration of protecting antibodies The improved counter immunoelectrophoretic method described recently (9) is compared with the toxin neutralization test in mice in the present study

Hitherto the passive haemagglutination test has been used for screening sera for tetanus antitoxin content at the National Institute of Public Health Oslo Also this test is compared with the toxin neutralization test in mice in this study

MATERIAL AND METHODS

Counter immunoelectrophoresis Standardization of the counter immunoelectrophoretic method has been described previously (9)

of an toxin kills a mouse of a defined weight in four days) The L+/10 toxin level titration was used for sera with high antitoxin content (≥ 3 I U /ml) The L+/400 toxin level titration was used for sera with low titres (≤ 5 I U /ml) The toxin used for these tests was precipitated with ammonium sulphate dried over P_2O_5 and stored in sealed ampoules Before use it was dissolved in 0 9 per cent saline containing 67 per cent glycerol

For titration at the L+/10 toxin level the sera were diluted in phosphate buffered saline pH 7 4 mixed with toxin and incubated at 20°C for 1 h 0 5 ml of each mixture was injected subcutaneously into an outbred female mouse (NMRI/Bom strain) weighing 16–18 g One mouse was used for each serum dilution The mice were observed for four days and deaths were recorded each day The lowest serum dilution causing the death of a mouse was taken as containing 0 1 I U /volume injected of tetanus antitoxin The International Standard for Tetanus Antitoxin 2nd Standard 1969 (1) obtained from Statens Seruminstitut Copenhagen was used as reference

Titration at the L+/400 toxin level was carried out according to a method introduced by *Ipsen* (4) by which the antitoxin titre is calculated on the basis of the time of death of the mice The same breed and size of mice were used as in the L+/10 toxin level titration method

Passive haemagglutination This was carried out essentially as described by *Stavitsky* (7) Sheep erythrocytes were formalinized and treated with tannic acid as described by *Butler* (2) Different partly purified tetanus toxoid preparations containing about 300 Lf/ml were used for sensitization of the erythrocytes Rabbit serum (0 5 per cent) was used as stabilizer in the final suspensions The International Standard for Tetanus Antitoxin (1) was used as reference The unknown sera were measured against the reference The antitoxin content was expressed in haemagglutination units (H U) 1 H U corresponded to 1 I U of the reference in the haemagglutination assay

Sera The serum specimens were tested within two months of collection About half of the specimens were taken from persons vaccinated within the previous three months while the other half were from persons vaccinated several years previously The age of the persons ranged from one to 60 years

Serum specimens found to be negative by counter immunoelectrophoresis were retested after concentration in Minicon S 125 (Amicon Corporation US A) concentrator or after lyophilization

Presentation of results Thirty sera were tested three times by counter immunoelectrophoresis using the respective toxoid preparations described previously (9) The same sera were tested three times in the toxin neutralization assay at the L+/10 toxin level The values recorded are the arithmetical means of the results In addition a further ten sera were tested once in the toxin neutralization assay at the L+/400 toxin level The same ten sera were tested by counter immunoelectrophoresis after concentration to one fifth Five sera were concentrated by lyophilization and five by Minicon S 125

143 sera were tested once by passive haemagglutination The same sera were tested once by toxin neutralization assay at the L+/400 toxin level

RESULTS

Comparison between anti tetanus antibody titres measured by counter-immunoelectrophoresis toxin neutralization test and passive haemagglutination
In order to investigate whether the anti tetanus IgG antibodies detected by counter immunoelectrophoresis could be responsible for the neutralization of tetanus toxin in mice the correlation coefficient between the counter immunoelectrophoresis titres

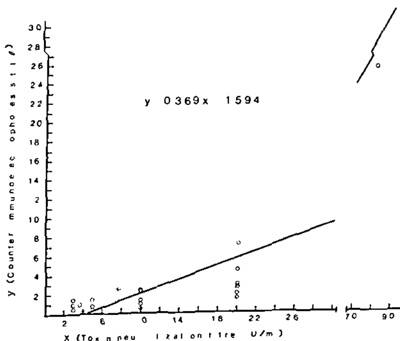
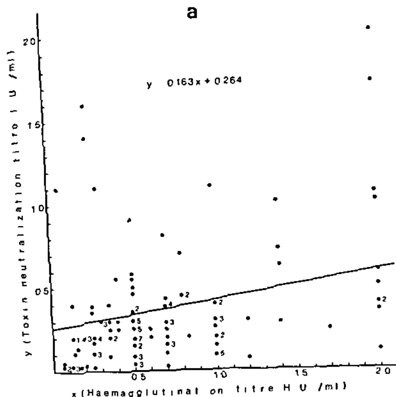


Fig 1 Scatter diagram of the relationship of counter immunoelectrophoresis titres to toxin neutralization titres The line drawn represents the regression equation between the variables calculated by the method of least squares (5) The symbol followed by a 2 represents two test results



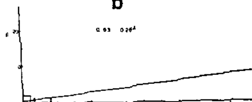


Fig 2 Scatter diagrams of the relationship of toxin neutralization titres to passive haemagglutination titres The lower titres are given in (a) and the higher titres in (b) The lines drawn represent the regression equation between the variables from both diagrams calculated by the method of least squares (5) The figures given after the dots represent the number of test results

The sensitivity of the counter immunoelectrophoretic method is not as good as that of the other two methods By the toxin neutralization test a minimum antitoxin amount of 0 003 I U /ml could be quantified According to the regression equation (Fig 1) antitoxin amounts of 7 0 I U /ml could be analysed in unconcentrated sera by counter immunoelectrophoresis After fivefold concentration of sera antitoxin amounts of 4 6 I U /ml could be assayed Antitoxin titres obtained by counter immunoelectrophoresis of 10 sera concentrated by lyophilization (5) or by ultrafiltration (5) correlated well with the titres obtained for the sera concerned by the toxin neutralization test in mice Since only 10 sera were analysed after concentration the correlation coefficient is not given

In repeated tests using counter immunoelectrophoresis and the toxin neutralization test the results varied within ± 33 per cent

and titres found by the toxin neutralization test was calculated for 30 sera (Fig 1) The correlation coefficient between the two was 0 89 by non logarithmic plotting and 0 76 by logarithmic plotting (5) This was considerably higher than the correlation coefficient of 0 76 between the titres obtained by toxin neutralization and passive haemagglutination by non logarithmic plotting (Figs 2a b) and of 0 50 by logarithmic plotting The correlation coefficients were highly significant according to Student s t test

DISCUSSION

Previous studies (9) indicated that counter immunoelectrophoresis may be used for quantification of human tetanus antitoxin For detection of tetanus antitoxin the accuracy of the method in repeated

analyses is comparable with the toxin neutralization test in mice The sensitivity of the *in vitro* method could be improved by concentration of sera without giving noticeably less well correlated results In the toxin neutralization test only antibodies of the IgG class seem to be effective (6) IgG antibodies could be quantified separately by counter immunoelectrophoresis A correlation coefficient between the two methods of 0 89 indicated that the same antibody populations were measured

Differences between the titres of antitoxins determined by passive haemagglutination and by toxin neutralization in animals have often been reported (reviewed by *van Ramshorst* (8)) In our hands also passive haemagglutination gave poor correlation with the toxin neutralization test in mice As reported in the literature (6) human antibodies of the IgG IgM and IgA classes react with tetanus toxoid in the passive haemagglutination test About half of the serum specimens analysed were taken within three months of vaccination Antitoxins of the IgM and the IgA class which are not limited to the primary immune response (3) may therefore have contributed to the results obtained by passive haemagglutination Contamination of the toxoids with extraneous antigens may also have had some effect

The conclusion to be drawn from the present study must however be that counter immunoelectrophoresis may be an advantageous substitute for passive haemagglutination in screening sera containing a relatively high amount of tetanus antitoxin since it is more well defined and less time consuming

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The skilled technical assistance of *Lill Ann Simonsen* is gratefully acknowledged

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ISOLATION OF *SPOROTHRIX FUNGORUM* FROM A 500-YEAR-OLD MUMMY FOUND IN GREENLAND

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Bodenhoff J Geertinger P & Prause J Isolation of *Sporothrix fungorum* from a 500 year-old mummy found in Greenland Acta path microbiol scand Sect B 87 201-203 1979

A 500 year-old mummy of a seven month old Eskimo infant recently removed from a tomb north of Umanak presented problems of preservation Among these was fungal coating on the skin especially on the face The fungus was isolated and diagnosed as a *Sporothrix fungorum* The skin was successfully treated with nystatine The species *Sporothrix fungorum* isolated is considered to be very rare having been reported previously only from Europe and South Africa but never from Greenland

Key words *Sporothrix fungorum* Greenland mummy

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The purpose of this paper is to report the isolation of the rare microorganism *Sporothrix fungorum* (de Hoog & de Vries 1973 de Hoog 1974) from an equally rare source – a 500 year-old Greenland mummy

MATERIAL AND METHODS

An Esk mo – –

style of the skin clothing the mummy is dated at 1460 ± 50 years A D

Specimens were taken consisting of scrapings from the abdomen and face of the mummy and from the inside of the skin clothing

Routine microscopical examination was performed on wet specimens Aerobic incubation was carried out at 25°C and at 37°C on corn meal agar (using *Dalmau*s technique (1929)) and on S

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stained for fungi with Grocott s modification of Gomori s methanamine silver nitrate technique

RESULTS

Microscopy of paraffin sections showed microorganisms with a close resemblance to fungi (Fig 1) Initial microscopy of scrapings also showed microorganisms resembling fungi (Fig 2)

Culture on Sabouraud agar incubated aerobically at 25°C gave mixed growth of *Torulopsis candida* and *Sporothrix fungorum* The latter diagnosis was primarily suggested to us by Drs *de Hoog* Rodriques de Miranda and *de Vries* of the Centraalbureau voor Schimmelcultures Baarn The Netherlands

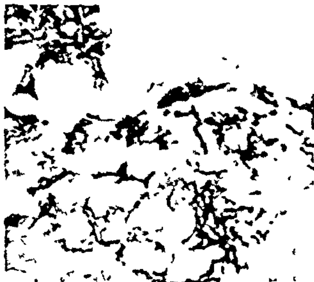


Fig 1 Paraffin section of sclera showing microorganisms resembling fungi (Grocott) × 400



Fig 2 Initial microscopy showing microorganisms resembling fungi

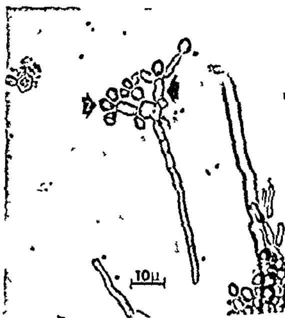


Fig 3 Sporothrix fungorum corn meal culture Conidial apparatus Arrow 1 primary conidia arrow 2 secondary conidia

Sporothrix fungorum grew slowly on all the agar types tested when kept aerobic at 25°C but no growth could be detected at 37°C

On Sabouraud agar (pH 4 5) the macroscopical appearance of the colonies was at first smooth and convex and a diameter of 2 5 to 4 mm was reached after incubation for 10 days The colonies were chalk white dull dry and opaque with a sharply defined slightly undulated margin There was no odour or exudation When airborne hyphae appeared (day 4) the colonies resembled small icebergs The reverse of the substrate was cream-coloured

Routine bright field observations of the corn meal agar by Zeiss photomicroscope showed submerged irregular hyphae and regular hyaline smooth walled vegetative hyphae with a diameter of 2 to 3 μm After culture for 48 hours cylindrical conidiogenous cells with small denticles were observed originating apically from non-differentiated hyphae

The sympodial growth took the form of both primary large cylindrical single-celled conidiae with apiculated base and secondary small blastic conidiae of a different shape originating from the conidiogenous cells (Fig 3)

Few septae were seen in young hyphae and their number increased with maturation of the conidiae When primary conidiae had formed on the hyphae lateral branches appeared just below the septae at sharp angles to the main stalk

The *Sporothrix fungorum* isolated was resistant

Fig 4 Head of 500 year old mummy found in Greenland Left side of the face treated with nystatine resulting in reduced fungal growth

to amphotericin B and miconazole but sensitive to the other antimycotics examined

Treatment of the mummy with a solution of nystatine clearly inhibited growth of fungi (Fig 4)

DISCUSSION

The differential diagnosis between the *Sporothrix fungorum* isolated and other closely related fungi such as *Ranularia Hyalodendron Cladosporium Blastobotrys nivea* etc is based primarily on the hyaline non-differentiated hyphae the cylindrical conidiogenous cells the sympodial conidiogenesis and the secondary conidiae which so clearly differ from the primary ones both in size and shape (*de Hoog* 1974)

Sporothrix fungorum has been isolated previously from three different localities only, viz in the Arberseewand Bavaria Germany where it was found in 1967 by *W Gams* in Vogelenzang The Netherlands, where *W Gams* isolated it in 1970 and at Savelsbos, South Africa where it was isolated by *J A Stalpers* in 1972 (*de Hoog & de Vries* 1973)

As the fungus is isolated only rarely the question is whether it is an inhabitant of Greenland today or whether it has remained in the mummies for 500 years This question can only be answered after careful investigations both at the site in Greenland and of the other mummies

The fact that a solution of an antimycotic to which the fungus is sensitive was found to inhibit the growth of the microorganism on the mummy and the skin clothing may be helpful in the conservation procedure

The authors wish to thank *G S de Hoog R de Miranda* [illegible] examine the mummy

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Acta path microbiol scand Sect B 87 205–209 1979

GAS CHROMATOGRAPHIC CHARACTERIZATION OF PORCINE AND HUMAN STRAINS BELONGING TO THE *MYCOBACTERIUM AVIUM-INTRACELLULARE* COMPLEX

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Larsson L Bergman R & Mårdh P A Gas chromatographic characterization of porcine and human strains belonging to the *Mycobacterium avium intracellulare* complex Acta path microbiol scand Sect. B 87 205–209 1979

Trifluoroacetylated whole cell methanolysates of 23 strains designated as belonging to the *Mycobacterium avium intracellulare* complex by biochemical growth chromogenicity and chicken pathogenicity tests were analysed by gas chromatography Twenty of the strains were isolated from pigs and the remainder from human beings Serological typing showed that 13 of the porcine strains but none of the human strains belonged to *M avium* The remaining strains except one which showed autoagglutination did not react with antisera to *M avium* (serotypes 1 3) thus suggesting that they belonged to *M intracellulare* Five different highly reproducible chromatographic patterns the main peaks of which were considered as representing bacterial carbohydrates and fatty acids, could be distinguished by visual examination and by cluster analysis The chromatographic results could not be correlated with those obtained from serotyping of the strains studied Mycobacteria recovered from different organs of one and the same pig gave virtually identical chromatograms The strains isolated from three human beings had a chromatographic pattern which was identical with one of those produced by the porcine strains The present investigation indicates that the gas chromatographic analytic technique used differentiates bacteria within the *M avium intracellulare* complex without assigning the organisms to species

Key words *Mycobacterium avium intracellulare* complex gas chromatography trifluoroacetyl derivatives

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Mycobacteria belonging to Runyon s group III constitute a group of non photochromogenic, slowly growing organisms which are virulent for a wide range of animal species and man (2 3 5 6 9 13 19–21 26) The difficulties encountered in differentiating some of the mycobacteria within this group are highlighted by their designation as »the *Mycobacterium avium intracellulare* complex« »*M avium* complex« and »avium like strains« (2 13 18 19 23 25 26)

Gas chrom— –

GC has been employed on mycobacterial fatty acids (11 15 22–24) derivatized whole-cell constituents (10 14) and pyrolysis products of lyophilized mycobacterial cells (16 17) Mycobacteria may be distinguished on a species level by the chromatographic patterns so obtained Intraspecies differences have also been demonstrated (16 23)

The present investigation was undertaken in order to study strains belonging to the *M avium intracellulare* complex by means of GC analysis of trifluoroacetyl (TFA) derivatives of whole-cell methanolysates GC analysis is considered to be a tool with which to differentiate these mycobacteria

MATERIALS AND METHODS

Microorganisms

The study comprised nineteen strains isolated from kidneys liver lungs and lymph nodes of 16 pigs and found to belong to the *M avium intracellulare* complex by biochemical and growth chromogenicity testing and by virulence tests in chicken (1) Sampling sites and designation of the strains studied are shown in Fig 2 In addition one reference strain of porcine origin (*M avium* NCTC 8551) and three human isolates belonging to the *M avium intracellulare* complex (kindly supplied by Dr *A Wickman* SBL Stockholm) were analysed Two of the latter strains were recovered from children with lymphadenitis and the remainder from a child with osteitis

Serotyping of the 23 strains studied was made in order to differentiate strains belonging to *M avium* (serotypes 1–3) from strains of *M intracellulare* The procedures were performed according to the method of *Schaefer* (20) The three sera used were kindly supplied by Dr *J Kenneth McClatchy* (National Jewish Hospital and Research Center Denver Colorado USA) and represented Schaefer s serotypes 1 and 3 (titres 1 160–320) and 2 (1 80–160) Four of the 20 porcine strains belonged to serotype 2 and 9 to serotype 3 (Fig 2) Nine of the strains including the three human isolates did not agglutinate any of the three sera used and one strain showed spontaneous agglutination Thus the serotyping suggested that 13 of the 23 strains belong to *M avium* and the remaining strains except one belong to *M intracellulare*

Culture Technique

Each of the strains examined was cultured on two slants of Lowenstein Jensen medium for 20 days at 37°C before inoculation into 100 ml of Proskauer Beck medium (Difco) These latter cultures were incubated for 30 days at 37°C One of the porcine strains was inoculated into six aliquots of Proskauer Beck medium and used in reproducibility tests The broth cultures were autoclaved and centrifuged at 4000 rev/min for 20 min The deposits were washed twice in distilled water before being lyophilized Prior to use the bacteria were stored under gaseous nitrogen at −20°C When testing *M avium* NCTC 8551 bacteria from a previous preparation were used (10)

Preparation of Bacteria for GC

1–2 mg of the lyophilized cells was transferred to 0 5 ml glass ampoules and dried overnight with phosphorous pentoxide in a vacuum desiccator 100 µl of *n* hexane containing 0 18 mg of tridecanoic acid was added as an internal standard and the sample was …orated to dryness by a stream of dry nitrogen

The ampoules were then sealed by [illegible] an oven at 80°C for 15 h After centrifugation (3000 rev/min for 15 min) the supernatant fluid was transferred to a 1 ml glass tube and concentrated to dryness after which ethyl acetate (20 µl) and trifluoroacetic anhydride (50 µl) were added After heating at 80 °C for 5 min followed by 20–30 min at room temperature 1–2 µl of the sample was injected into the gas chromatograph

Gas Chromatograph

The apparatus and GC conditions used were those described previously (10) In all experiments a 3% OV 101 stationary phase on Chromosorb W HP 80–100 mesh was used The temperature of the column was programmed from 80 to 280°C using a temperature increase of 6 min ⁻¹

Test of Reproducibility and Procedures of Numerical Analysis

A Varian chromatographic data system (CDS 101) was used for calculation of peak areas The standard deviations of the areas of six major peaks present in the chromatograms of all strains studied were determined by analysis of the six different cultures of one of the strains The mean values of the major peak areas were calculated from duplicate cultures of each of the 20 porcine strains tested These values were used for cluster analysis (7) based on Euclidean distances (4)

RESULTS

GC Elution Profiles

All 23 strains studied yielded similar chromatographic patterns although quantitative differences could be distinguished as regards the areas of certain peaks present in all chromatograms (Fig 1) Particular interest centred on six of the peaks with approximate retention times of 4 5 (retention ratio related to the internal standard 0 35) 7 5 (0 58) 18 5 (1 42) 21 5 (1 65) 22 0 (1 69) and 22 5 (1 73) min These peaks marked *a* to *f* and that of the internal standard (s) representing tridecanoic acid methyl ester constitute the major peaks in the chromatograms On visual examination five distinct chromatographic patterns could be distinguished

Representative chromatograms of these five groups are shown in Fig 1 Groups A B and C are characterized by approximately equal *a* and *b* peak sizes but relative heights of peaks *d f* were different Three of the porcine strains produced chromatograms of type group A which were characterized by a larger *e* than *d* peak Group B (3 strains) had a larger *f* than *d* peak and the chromatograms of group C (1 strain) had a larger *d* than *f* peak In groups D (12 strains) and E (1 strain) the relative distributions of the sizes of peaks *d*–*f* were similar to those of group B though with a larger (group D) and still larger (group E) peak *b* than peak *a* respectively

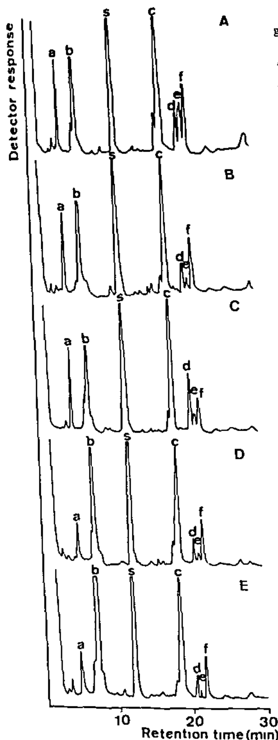


Fig 1 Five different chromatographic patterns obtained by gas chromatographic analysis of trifluoroacetylated whole-cell methanolysates of 23 strains belonging to the *Mycobacterium avium intracellulare* complex See text for explanation of symbols a–f and A–E s – internal standard of tridecanoic acid methyl ester

The three human strains all produced chromatograms of type group D

Reproducibility of Chromatograms

Analysis of one of the strains belonging to group D (strain 22) which had been cultured on six aliquots of Proskauer Beck medium revealed that the standard deviations of the six major peak areas were 0 87 (mean relative peak area 15%) 0 74 (43%) 0 47 (20%) 0 44 (7%) 0 55 (3%) and 0 75 (12%) respectively Chromatograms obtained from the two analyses of each strain tested were always superimposable

Cluster Analysis

Fig 2 shows a phenogram based on the mean peak areas of the six major peaks obtained from the two analyses of each of the 20 porcine strains studied At an amalgamated distance between 4 6 and 8 7 (dotted lines in the figure) the strains could be separated into five groups (A–E) corresponding to those observed by visual examination of the chromatograms Differences between the strains at a lesser distance could not be related to any distinct differences when the chromatograms were compared visually

Chromatograms of groups A B and D all contained strains of different serotypes Organisms within A and B belonged to *M avium* while group D contained both *M avium* serotypes 2 and 3 and *M intracellulare*

Strains isolated from different organs of one and the same pig all belonged to *M avium* serotype 3 and were chromatographically identical at an amalgamated distance of 4 6 (Fig 2)

DISCUSSION

Mycobacteria of the *M avium intracellulare* complex are the cause of infections affecting mainly birds and mammals (3 5 19 21) They usually cause local infections although cases of generalized infections do occur In many countries infections with these organisms which respond poorly to chemotherapy are responsible for serious economic losses in the m [illegible]

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are [illegible] to be transmitted by animals (5) However the unsatisfactory means of differentiating these mycobacteria is one obstacle to epidemiological studies In order to elaborate more reliable and rapid characterization techniques than those obtained by pathogenicity and growth chromogenicity testing immunological methods and analysis of

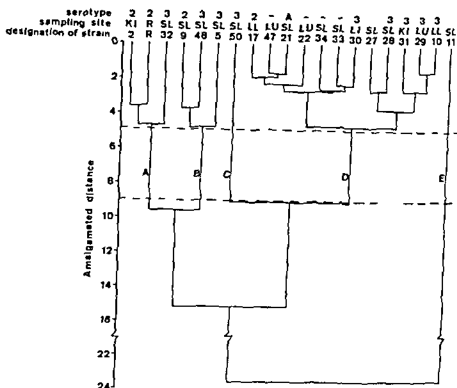


Fig 2 Phenogram obtained by cluster analysis of mean values of six prominent peak areas from gas chromatograms of analysis of 20 porcine strains belonging to the *Mycobacterium avium intracellulare* complex. The results of serotyping *using antisera to serotypes 1–3 are indicated. Strains marked with (–) did not agglutinate any of the three* antisera used, while (A) expresses autoagglutination. *Strains designated 28–31 were all recovered from the same pig.* Symbols for sampling site: LU = lung, LL = lung lymph node, SL = submandibular lymph node, LI = liver, KI = kidney *and* R = *reference strain (NCTC 8551). Numerals express the designation of the strains tested.*

the cellular lipids by thin layer chromatography (TLC) have been employed. However, some strains yield indistinct TLC patterns which are non-*interpretable (»non typeable«) even when large* quantities of bacteria are used (1, 2, 12). In serotyping, strains may fail to agglutinate available antisera, while others tend to autoagglutinate or agglutinate sera of more than one serotype (2, 5, 19, 26).

The value of GC in the differential diagnosis of mycobacteria is well documented (10, 11, 14–17, 22–24). We have previously carried out GC analyses of both mycobacterial fatty acids and TFA *derivatized mycobacterial whole-cell methanolysates.* The latter technique was found to give chromatograms providing more information on the chemical composition of the bacteria studied, since also carbohydrates, analysed as TFA derivatized methyl glycosides, are included (8, 10). Mycobacterial strains of the same species but isolated from different human individuals gave chromatograms which were virtually identical, while several freshly isolated strains of the different species studied, viz. *M. bovis* strain BCG, *M. kansasii* and *M. tuberculosis* and *M. avium* strain NCTC 8551 gave distinguishable chromatographic patterns (10). The differences obtained in the present study between TFA chromatographic elution profiles representing various strains of *M. avium* and *M. intracellulare* were as clear-cut as between those of the four species tested previously. The chromatographic differences are therefore considered to reflect definite intraspecies differences in the chemical composition of the strains of the *M. avium intracellulare* complex studied.

The remarkable reproducibility of TFA chromatograms representing bacterial cellular constituents reported earlier (8, 10) was confirmed in the present *investigation. The use of tridecanoic acid* as internal standard was found valuable as a control of the prevailing GC conditions.

A *heterogeneity of cellular fatty acids of* various serotypes of mycobacteria of the *M. avium intracellulare* complex as studied by GC has been *reported previously (23) even among strains of* the same serotype. These findings were supported by the present study. Using the GC technique, *M. avium could not be distinguished from M. intracellulare.* On the other hand, both species were found to include organisms with a chemical heterogeneity

which was comparable with that obtained in studies on mycobacteria of different species

This study was supported by the *Swedish National Association against Heart and Chest Diseases* We extend grateful thanks to Dr *Bo Gullberg* for performing the cluster analysis and to Dr *Anders Gunnarsson* for guidance in the serological studies

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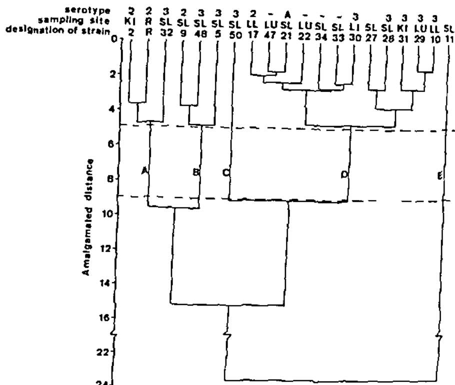


Fig 2 Phenogram obtained by cluster analysis of mean values of six prominent peak areas from gas chromatograms of analysis of 20 porcine strains belonging to the *Mycobacterium avium intracellulare* complex The results of serotyping using antisera to serotypes 1–3 are indicated Strains marked with (–) did not agglutinate any of the three antisera used while (A) expresses autoagglutination Strains designated 28–31 were all recovered from the same pig Symbols for sampling site LU – lung LL = lung lymph node SL = submandibular lymph node LI ≈ liver KI = kidney and R – reference strain (NCTC 8551) Numerals express the designation of the strains tested

the cellular lipids by thin layer chromatography (TLC) have been employed However some strains yield indistinct TLC patterns which are non interpretable (›non typeable«) even when large quantities of bacteria are used (1 2 12) In serotyping strains may fail to agglutinate available antisera while others tend to autoagglutinate or agglutinate sera of more than one serotype (2 5 19 26)

The value of GC in the differential diagnosis of mycobacteria is well documented (10 11 14–17 22–24) We have previously carried out GC analyses of both mycobacterial fatty acids and TFA derivatized mycobacterial whole-cell methanolysates The latter technique was found to give chromatograms providing more information on the chemical composition of the bacteria studied since also carbohydrates analysed as TFA derivatized methyl glycosides are included (8 10) Mycobacterial strains of the same species but isolated from different human individuals gave chromatograms which were virtually identical while several freshly isolated strains of the different species studied viz *M bovis* strain BCG *M kansasii* and *M tuberculosis* and *M avium* strain NCTC 8551 gave distinguishable chromatographic patterns (10) The differences obtained in the present study between TFA chromatographic elution profiles representing various strains of *M avium* and *M intracellulare* were as clear-cut as between those of the four species tested previously The chromatographic differences are therefore considered to reflect definite intraspecies differences in the chemical composition of the strains of the *M avium intracellulare* complex studied

The remarkable reproducibility of TFA chroma ... cellular constituents ... nt ... nal standard was found valuable as a control of the prevailing GC conditions

A heterogeneity of cellular fatty acids of various serotypes of mycobacteria of the *M avium intracellulare* complex as studied by GC has been reported previously (23) even among strains of the same serotype These findings were supported by the present study Using the GC technique *M avium* could not be distinguished from *M intracellulare* On the other hand both species were found to include organisms with a chemical heterogeneity

Acta path microbiol scand Sect B 87 211–216 1979

CHARACTERIZATION OF FRACTIONS CONTAINING THE THERMOSTABLE AGGLUTINOGENS OF *NEISSERIA GONORRHOEAE*

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Eggset G Iversen O J & Mæland J A Characterization of fractions containing the thermostable agglutinogens of *Neisseria gonorrhoeae* Acta path microbiol scand Sect B 87 211–216 1979

Extract was prepared by heat treatment (100°C 2 h) of bacteria of N gonorrhoeae strain 8551 The acid precipitate (pH 2 5) of the extract was resolved by gel chromatography (Sepharose CL 6B) into material eluted with the void volume (Fr I) and material retarded by the column (Fr II) Fr I con[illegible]

b [illegible]

ext [illegible]

precipitinogens The antigens of Fr I included the heat stable agglutinogens of the gonococcus and antibodies to these could be raised by immunization of rabbits with the Fr I material

Key words *Neisseria gonorrhoeae* thermostable agglutinigens characterization

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Antibody mediated agglutination of heated cells of Gram negative bacteria is referred to as O agglutination This designation accords with the concept that the agglutination is caused solely by antibody to the O antigen i e the lipopolysaccharide (LPS) of the bacterium Recently this assumption was questioned in the case of *Pseudomonas aeruginosa* (4) In gonococci LPS functions as an agglutinogen (18) but in tests using heated cells of the bacteria and antiserum to whole bacterial cells LPS was not the only agglutinoten (1) All the thermostable agglutinogens were present in the bacterial heat extract (1)

The outer membrane of *N gonorrhoeae* cells like that of other Gram negative bacteria contains LPS and proteins (5 7) At least one of the envelope protein antigens is thermostable (13) Thus it is possible that thermostable proteins of the outer membrane may function as agglutinogens in heated gonococci If so the agglutination test may [illegible] detection of both protein and LPS serotype antigens of the bacterium

This report describes the results of a study designed to characterize the antigens of the gonococcal heat extract Particularly fraction containing the thermostable agglutinogens of the bacterium was studied

MATERIAL AND METHODS

Strain

N gonorrhoeae strain 8551 (15) colony type T4 was cultured and harvested as previously (1)

Preparation of Antigens

Thermostable antigens Heat extract (2 h 100°C) of the gonococcus was prepared as reported earlier (1) The extract was acidified with HCl to pH 2 5 and kept at 4°C for 20 h The precipitate formed was collected by centrifugation dissolved in phosphate buffered saline pH [illegible] (S) and again precipitated at pH 2 5 The final

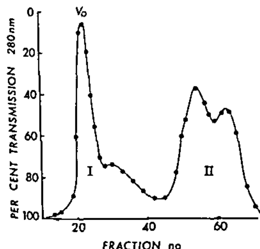


Fig 1 The elution profile of 20 mg of the acid predipitate of the gonococcal heat extract chromatographed on a Sepharose CL-6B column V_0 represents the void volume of the column

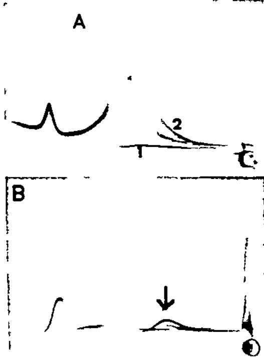


Fig 3 Crossed immunoelectrophoresis (A) Fr I material dissolved in the NaD buffer and tested against the anti Fr I serum diluted 1 40 (B) Fr II material dissolved in the elevtrophoresis buffer and tested against the anti Fr II serum diluted 1 12 Arrow indicates the precipitation line lost on absorption of the anti Fr II serum with Fr I Anode to the left.

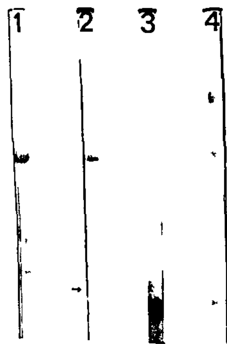


Fig 2 SDS-polyacrylamide gel electrophoresis of Fr I (1) LiAP (2) Fr II (3) and protein standards (4) The gels were stained for proteins with Coomassie brilliant blue Arrow indicates a protein band in LiAP not visible on photograph

SDS polyacrylamide gel electrophoresis showed that Fr I conta ned at least s . . (F . .

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Immunoprecipitation

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c . . o the anode when the antiserum was used in more concentrated form At that concentration separation of lines 1 and 2 was not obtained A similar pattern of immunoprecipitates was obtained with the anti-Gc 8551 or anti LiAP serum No line was formed when using anti Fr I serum absorbed with Fr I or with untreated or heated gonococci

precipitate was dissolved in a 0 05 M tris HCl buffer pH 8 0 with 0 14 M NaCl Up to 30 mg of the material (10 mg per ml) was passed through a column (2 5 × 80 cm) of Sepharose CL 6B (Pharmacia Fine Chemicals Uppsala Sweden) stabilized and eluted with the tris HCl buffer at a flow rate of 0 2 ml per min Fractions of 6 ml were collected and monitored at 280 nm Peak fractions were pooled dialysed against water and lyophilized The void volume of the column was measured with Blue Dextran (Pharmacia)

Lithium acetate preparation (LiAP) The preparation method used was as described by *Johnston et al* (7) Briefly whole gonococcal cells were extracted with 0 2 M lithium acetate pH 6 0 with 0 010 M EDTA The high molecular weight material (LiAP) of the extract was isolated by ultracentrifugation and gel filtration (Sepharose CL 6B) as reported previously (7)

Lipopolysaccharide (LPS) and determinants a *and* b LPS was extracted from bacterial whole cells with phenol water and purified by ultracentrifugation (11) The carbohydrate determinant *a* of LPS was prepared as described previously (15) The protein determinant *b* of the gonococcus was prepared by extraction of bacterial whole cells with alkali (10)

Serological Methods

Crossed immunoelectrophoresis (CIE) CIE was performed essentially as described by others (21) Agarose (Litex Glostrup Denmark) 1 per cent was prepared in veronal tris buffer pH 8 6 (4 5 g of veronal 8 85 g of tris 0 108 g of Ca lactate 0 128 g of N azid per 1000 ml H_2O) and the electrophoresis run in the same buffer Material was dissolved in the veronal tris buffer or in an endotoxin disaggregating buffer (NaD buffer) containing Na deoxycholate (8) and 0 25 mg was applied to the antigen well The antibody containing gel was mixed with suitable dilutions of rabbit antiserum After electrophoresis the gels were processed and stained for proteins with Coomassie brilliant blue (20)

Immunodiffusion The test was performed as described previously (3) using double diffusion slides prepared with 1 per cent agarose in PBS The wells were filled with 0 15 mg of the antigen (10 mg/ml) dissolved in the NaD buffer or with undiluted antiserum The gels were stained for proteins with Coomassie brilliant blue (20)

Bacterial agglutination test The titre of the antiserum against heated cells of the gonococcus was determined using the technique described previously (1)

Indirect haemagglutination Sheep erythrocytes were sensitized with determinant *a* or *b* as reported earlier (11) The haemagglutination assay was performed as described previously (11) except that microtitre equipment (Cooke Laboratory Products Aleksandria Virginia) was used and the results were recorded by observing the pattern formed by the sedimented erythrocytes

Absorption of antiserum Antisera were absorbed with untreated or heated gonococci as reported previously (1) Absorption was preformed with 20 mg of the acid precipitate of the heat extract or 10 mg of the material eluted from the Sepharose CL 6B column per ml of undiluted antiserum The mixtures were kept at 4°C for 24 h and centrifuged at 12000 × *g* for 15 min after which the supernatant fluid was examined

Other Methods

SDS polyacrylamide gel electrophoresis Electrophoresis was performed as described by *Weber & Osborn* (19) using 10 per cent (w/v) acrylamide and 0 1 per cent sodium dodecyl sulphate (SDS) in a 0 1 M phosphate buffer pH 7 0 (19) Samples (5 mg per ml) were incubated for 2 h at 37°C in 0 01 M phosphate buffer containing 1 per cent SDS and 1 per cent 2 mercaptoethanol and 40 µl was applied to the gel Cytochrome C (12 500 daltons) chymotrypsinogen (25 000 damtons) bovine serum albumin (67 000 daltons) and aldolase (158 000 daltons) purchased from Boeringer Mannhein West Germany were used as standards The tracking dye was bromphenol blue SDS was removed by repeated washings of the gels with 7 per cent acetic acid before staining for proteins with Coomassie brilliant blue (19) or for carbohydrates with Alcian blue (9)

Immunization Rabbit antiserum to whole gonococcal cells (anti Gc 8551) was obtained as in a previous study (1) Lyophilized material from the bacterium was suspended in PBS (1 mg per ml) and used for immunization of rabbits one dose being injected each week Doses of 0 4 0 8 and 0 8 mg were injected intracutaneously and the 1 mg intramuscularly until testing of serum by bacterial agglutination and immunodiffusion showed satisfactory antibody response The antisera obtained were designated anti Fr I Fr II and LiAP according to the preparation used for immunization

RESULTS

Fractionation and Gel Electrophoresis

Acidification of the heat extract of the gonococcus to pH 2 5 resulted in precipitation of approximately 50 per cent of the extract material When this material was used for absorption of the anti Gc 8551 serum all antibody activity detectable by the bacterial agglutination test was removed Material of the supernatant fluid after the acid precipitation had no such effect

Gel filtration on a Sepharose CL 6B column resolved the acid precipitate into material (Fr I) eluted with the void volume and material (Fr II) retarded by the column (Fig 1) All the material of LiAP showing absorbance at 280 nm eluted at the void volume of the column

The lyophilized Fr II material dissolved readily in water or buffers without a detergent Fr I material was soluble in the NaD buffer but was only partially soluble in buffers without a detergent LiAP dissolved poorly even in the NaD buffer This preparation was therefore unfit for use as antigen in serological testing

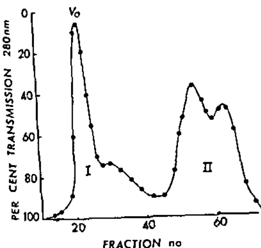


Fig 1 The elution profile of 20 mg of the acid precipitate of the gonococcal heat extract chromatographed on a Sepharose CL-6B column V_0 represents the void volume of the column

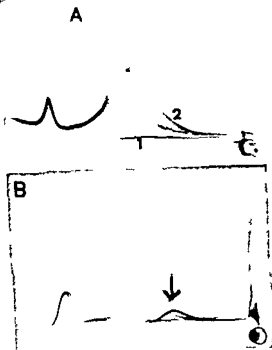


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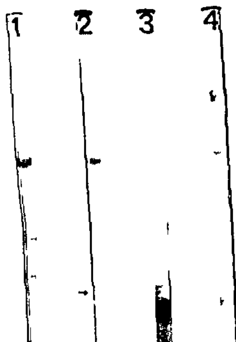


Fig 2 SDS polyacrylamide gel electrophoresis of Fr I (1) LiAP (2) Fr II (3) and protein standards (4) The gels were stained for proteins with Coomassie brilliant blue Arrow indicates a protein band in LiAP not visible on photograph

SDS polyacrylamide gel electrophoresis showed that Fr I contained at least six proteins (Fig 2) and LiAP at least four proteins all of which corresponded to protein lines in Fr I The molecular weight of the major protein of both preparations was 60 000 daltons Fr II contained several proteins When stained for carbohydrates all three preparations showed a band with mobility slightly higher than that of cytochrome C

Immunoprecipitation

CIE testing of Fr I against the anti Fr I serum showed the development of the immunoprecipitates 1 and 2 (Fig 3A) A third precipitate was observed close to the anode when the antiserum was used in more concentrated form At that concentration separation of lines 1 and 2 was not obtained A similar pattern of immunoprecipitates was obtained with the anti-Gc 8551 or anti LiAP serum No line was formed when using anti Fr I serum absorbed with Fr I or with untreated or heated gonococci

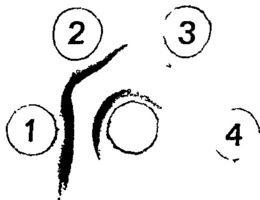


Fig 4 Immunodiffusion in agar gel Fr I material (central well) tested against anti Gc 8551 (1) Fr I (2) LiAP (3) and Fr II (4) The gel was stained with Coomassie brilliant blue

When this antiserum was absorbed with Fr II or LPS precipitate 2 was no longer demonstrable

Several precipitation lines developed when testing Fr II against the anti Fr II serum (Fig 3B) When the antiserum was absorbed with Fr I or LPS one of the lines was no longer produced (see Fig 3B) Absorption with bacteria obviated the formation of all except one precipitate located at the borderline between the upper and lower gels

In the agar gel diffusion test anti Gc 8551 Fr I and LiAP showed similar precipitation line patterns when tested against the Fr I material (Fig 4)

Indirect Haemagglutination and Bacterial Agglutination

Agglutination of erythrocytes sensitized with the determinant *a* or *b* was obtained with anti Gc 8551 -Fr I and LiAP (Table 1) Anti Fr II showed antibody activity against determinant *a* but not against *b* Corresponding pre immune sera showed titres less than 8 against these antigens In

TABLE 1 *Titre of Antisera Tested Against Erythrocytes Sensitized with Determinant* a *or* b

Antiserum	Determinant *a*	*b*
Anti Gc 8551	256	128
Anti Fr I	256	64
Anti Fr II	128	<8
Anti LiAP	64	64

TABLE 2 *Titre in the Bacterial Agglutination Test of Antisera before and after Absorption with Various Materials*

Material used for absorption	Anti Gc 8551	Fr I	Fr II	LiAP
None	256	256	64	64
Gonococci	<8	<8	<8	<8
Fr I	<8	<8	<8	<8
Fr II	64	64	<8	32

agreement with these results antibody to the determinant *a* or *b* was removed by absorption of anti Gc 8551 with Fr I Absorption with Fr II removed the antibody to *a* but not to *b*

All the antisera examined agglutinated the gonococci (Table 2) Agglutination was not observed after absorption of the antisera with bacteria or the Fr I material Absorption with Fr II or LPS removed the agglutinins from the anti Fr II serum but not from the other antisera

The titres of the antisera were only insignificantly affected by absorption with LiAP probably due to its insolubility in the PBS serum mixture

DISCUSSION

The acid precipitate of the gonococcal heat extract was separated by gel filtration into material excluded by a Sepharose CL 6B column (Fr I) and material retarded by the column (Fr II) Both fractions contained several proteins and at least one polysaccharide CIE testing showed that Fr I contained two dominating highly heterogeneous precipitinogens 1 and 2 and at least one additional precipitinogen The results of testing using absorbed antisera indicated that precipitinogen 2 of Fr I was present also in Fr II and that this antigen corresponded to LPS of the bacterium The finding of LPS both as part of the heavily aggregated material of Fr I and as part of the unaggregated material of Fr II is in accordance with the observations reported by *Johnston* and may be related to the composition of the elution buffer (6)

LPS from gonococci may be associated with varying amounts of protein When prepared by the hot phenolwater method LPS contained very little protein (16 17) while protein dominated the LPS containing complex obtained with aqueous ether (13) This latter preparation contained the carbohydrate determinant *a* of LPS

determinant *b* (12) both originating from the bacterial cell envelopes (13) Indirect haemagglutination testing in this study showed that Fr I contained *a* and *b* Thus heat extraction like aqueous ether extraction resulted in the release of an LPS which was closely associated with proteins in high molecular weight aggregates The *a* determinant must be linked to precipitinogen 2 since line 2 did not develop on CIE testing of antiserum absorbed with LPS The *b* determinant may be linked to precipitinogen 1 but as yet this has not been established

Fr I contained components of the outer membrane of the gonococci as indicated by the detection of LPS which is a major outer membrane marker and by the results of comparative analysis of LiAP that has been considered to be a representative preparation of the outer membrane (6) All the LiAP proteins detected by gel electrophoresis and a polysaccharide presumably LPS corresponded to components in Fr I Moreover rabbit antiserum to LiAP contained antibodies to the determinants *a* and *b* and antibodies that resulted in precipitation patterns similar to those observed with anti Fr I It thus appears that the major antigens of Fr I may be identical with those of LiAP This strongly suggests enrichment of Fr I with outer membrane components

The insolubility of LiAP and the molecular weight of the major protein of this preparation are at variance with what has been reported previously (6 7) It is possible that this can be accounted for by strain differences *Johnston et al* (7) reported classification of gonococci into 16 serotypes based on a protein in LiAP Whether the serotype antigen is identical with the protein antigenic determinant *b* present in LiAP is not known LPS which is a carrier of serotype antigenic determinants (2 14 15) was also present in the LiAP Methods are required that can distinguish clearly between protein and LPS serotype antigens in the classification of gonococci as was emphasized recently in a study on type determination of meningococci (22)

Anti Fr II serum absorbed with Fr I did not agglutinate the gonococcus However the absorbed serum still contained an bod [illegible]

[illegible] not sensitive enough to detect these antibodies or that the corresponding antigens did not function as agglutinogens On the other hand Fr I contained at least the major heat stable aggl[illegible] [illegible] nic specificity has been demonstrated by the bacterial agglutination test using heated gonococci (1) The relationship between the serotype agglutinogen(s) of the bacterium and the antigens of Fr I and the possible use of antisera to this fraction in type classification of gonococci deserve clarification

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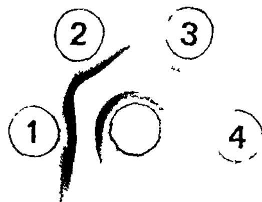


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Fr I	<8	<8	<8	<8
Fr II	64	64	<8	32

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DESTRUCTION OF *TOXOPLASMA GONDII* BY HCl SOLUTION

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Pettersen E K Destruction of *Toxoplasma gondii* by HCl solution Acta path microbiol scand Sect B *87* 217–220 1979

It was shown that the destruction of *Toxoplasma gondii* by pepsin HCl solution was due to the acid only Endozoites were destroyed faster by hydrochloric acid than cystozoites Furthermore endozoites were stained faster than cystozoites in an alkaline solution of methylene blue For this reason endozoites were assumed to have a higher permeability than cystozoites The action of hydrochloric acid was used for demonstration of cystozoites in mouse peritoneum cavity No cystozoites were found after inoculation of virulent parasites

Key words *Toxoplasma gondii* destruction by HCl solution virulence tissue cysts

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In 1960 *Jacobs et al* described a method for the isolation of *Toxoplasma gondii* from chronically infected animal tissue The tissue was incubated at 37°C in a pepsin HCl solution It was found that the integrity of cyst walls was destroyed immediately on contact with the digestive solution The released cystozoites (*Hoare* 1972) were found to be infective after incubation for two hours whereas endozoites (*Hoare* 1972) were destroyed after incubation in pepsin HCl solution for 30 minutes Consequently the action of pepsin HCl solution which was thought to be due to the enzyme became a means for distinguishing between endozoites and cystozoites (*Remington et al* 1960)

As endozoites survived incubation with trypsin for at least three hours (*Jacobs et al* 1960) it was considered of interest to see to what extent hydrochloric acid influenced the destruction of these parasites

In this paper the virulence of »virulent parasites« is equal to that of strain RH since they killed all mice regardless of the number of parasites inoculated »Avirulent parasites« will not kill the mice unless a large number of parasites is inoculated

MATERIAL AND METHODS

The parasites used in this investigation were of four different strains of *Toxoplasma gondii* The virulent strains 119 and RH killed all the mice inoculated The parasites were obtained from mouse peritoneal cavity four days after inoculation of 40 000 five days after inoculation of 20 000 and seven days after inoculation of 100 endozoites

Avirulent parasites of strains 178 and DUE were kept in mice by passage of mouse brains containing tissue cysts The brains were washed with sodium chloride solution and incubated with pepsin HCl solution After about five minutes at 37°C the brains were comminuted with a spatula and the suspension was neutralized by adding nine volumes of phosphate buffer Coarse particles were allowed to settle and the supernatant fluid containing the cystozoites was centrifuged at 500 x *g* for 15 minutes The sediment was suspended in a small volume of phosphate buffer and inoculated into mice each mouse receiving approximately two million cystozoites Endozoites were harvested from the mouse peritoneum cavity for investigation of acid lability

For investigation of acid lability and stainability with methylene blue cystozoites were purified by adding 10 per cent by volume of a rabbit antimouse immunoglobulin preparation to a suspension of the parasites After 30 minutes at room temperature most of the mouse cells

TABLE 2 *Destruction of Avirulent Parasites*

Time in minutes	Strain DUE (4) in HCl RT	Strain DUE (6) in HCl RT	Strain 178 (7) in pepsin HCl 37°C	Strain 178 (7) in HCl 37°C	Strain DUE cystozoites in pepsin HCl 37°C
30	0/2/3	0/4/1	0/5/0	1/4/0	5/0/0
60	0/0/5	0/3/2	0/5/0	0/3/2	5/0/0
90	0/0/5	0/1/4	1/3/1	0/4/1	5/0/0
120	0/0/5	0/0/5	0/2/3	0/1/4	5/0/0
150	0/0/5	0/0/5	0/0/5	0/0/5	5/0/0
180			0/0/5	0/0/5	5/0/0

Numbers in brackets indicate which day after inoculation the parasites were harvested
Figures indicate the number of mice died/infected/not infected (determined by the dye test)
RT means room temperature

were inoculated with acid treated parasites After two weeks blood was taken from surviving mice for examination by the dye test All the mice died when inoculated with strain DUE cystozoites even after incubation in pepsin HCl solution for three hours

Fig 1 shows the destruction per unit time of parasites incubated in HCl solution at room temperature The figures were obtained from direct microscopical counts of parasites surviving the incubation Curve A represents the destruction of parasites obtained from mouse peritoneum cavity four days after inoculation of strain RH endozoites Examination of parasites harvested seven days after inoculation of the same strain showed an identical rate of destruction

Curve B in the figure shows the destruction of parasites harvested four days after inoculation of strain DUE cystozoites Almost identical rates of destruction were obtained with parasites harvested six days after inoculation of the same strain and with parasites harvested seven days after inoculation of strain 178 cystozoites

Curve C in the figure indicates the rate of destruction of strain DUE cystozoites in HCl solution This was drawn on the basis of the result that half the cystozoites were destroyed after incubation for 150 minutes

The number of parasites found by the method described above was multiplied by six in order to obtain concentration of parasites per µl This was based on a comparison made on ten samples of a parasite suspension counted by the above method using a 0 1 mm Fuchs Rosenthal counting chamber Standard deviations for the two methods were 15 and 17 per cent of the counted numbers respectively

Endozoites of strain RH were completely stained by methylene blue within one minute of contact Cystozoites of strain 178 did not take the stain until 30 minutes after contact with the dye Only after 60 minutes of contact were these parasites stained completely

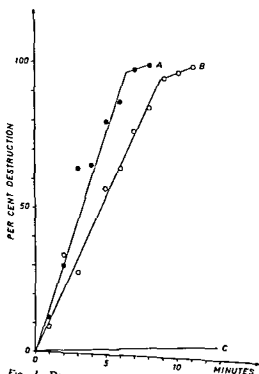


Fig 1 Destruction of *Toxoplasma gondii* in HCl solution at room temperature
A = Strain RH endozoites B = Strain DUE endozoites
C = Strain DUE cystozoites

were agglutinated and these were sedimented by centrifugation at 50 x *g* for 10 minutes The cystozoites were sedimented by centrifugation at 500 x *g* and washed once with phosphate buffer before use

Strains 119 and 178 were described in an earlier publication (*Pettersen* 1977) Strain DUE was isolated in 1958 from a pidgeon (*Sim* unpublished) and has since been kept in mice by passage of brain tissue once or twice a year

The destruction of *Toxoplasma gondii* was investigated by adding the same volume of a parasite suspension to a number of tubes The volume was adjusted so that each tube contained approximately 100 000 parasites The tubes were centrifuged for 10 minutes at 500 x *g* the supernatant liquids were removed and aliquots of 0 1 ml of either pepsin HCl solution or HCl solution were added The tubes were incubated for different times after which the contents were neutralized by adding 0 9 ml aliquots of phosphate buffer The content of each tube was inoculated into five mice

The destruction of *Toxoplasma gondii* by HCl solution was also investigated by direct count of the parasites remaining in a suspension at different times At zero time the suspension was made by stirring five million parasites into 0 5 ml of HCl solution At intervals of one minute 25 µl of the suspension was pipetted into 225 µl of phosphate buffer and 10 µl of the neutral suspension was placed on a slide The number of parasites appearing across an 18 × 18 mm cover slip was counted using a field diameter of 0 37 mm

Staining of *Toxoplasma gondii* was investigated by mixing a suspension of the parasites with an equal volume of 0 2 per cent methylene blue in borate buffer pH 11 (*Aagaard* 1960)

Rabbit antimouse immunoglobulin was prepared from serum of immunized rabbits (*Pettersen* 1967) by adding 25 per cent by weight of ammonium sulphate and stirring The precipitate was washed once with 25 per cent ammonium sulphate solution and dissolved in deionized water The solution was submitted to column dialysis (*Pettersen* 1972) first against deionized water and then against sodium chloride solution The final volume was approximately 10 per cent of the serum used

The mice used in this work were of strain A67 bred at Statens Seruminstitut At time of inoculation they weighed 18–20 g Serum from mice still alive two weeks after inoculation was investigated for specific antibodies by the *Sabin Feldman* dye test (1948) (*Aagaard* 1960)

The HCl solution contained 7 ml of concentrated hydrochloric acid and 5 g of sodium chloride per litre (*Jacobs et al* 1960) When required 3 000 units of pepsin (Sigma No P–7012) were added per ml of HCl solution

Sodium chloride solution was 0 15 M with respect to sodium chloride and 0 003 M with respect to sodium azide

The phosphate buffer was 0 05 M with respect to sodium phosphate 0 1 M with respect to sodium chloride and 0 003 M with respect to sodium azide The pH was adjusted to 7 4

RESULTS

Incubation of virulent parasites in HCl solution gave the results shown in Table 1 The numbers in brackets indicate the day after inoculation on which these parasites were obtained from the mouse peritoneum cavity The results show the number of mice that died survived with a positive dye test titre and survived with a reciprocal dye test titre <10 Five mice in each group were inoculated with the acid treated parasites Four weeks later there were no survivors with a positive dye test titre Most of the infected mice died within 12 days

Similar experiments with avirulent parasites gave the results shown in Table 2 Again the parasites were obtained from mouse peritoneum cavity on the day indicated in brackets Five mice in each group

TABLE 1 *Destruction of Virulent Parasites*

Time in minutes	RH strain (4) in pepsin HCl RT	RH strain (4) in pepsin HCl 37°C	RH strain (4) in HCl RT	RH strain (4) in HCl 37°C	RH strain (7) in HCl RT	Strain 119 (5) in HCl RT
5		5/0/0		5/0/0		
10	5/0/0	5/0/0	5/0/0	5/0/0	5/0/0	5/0/0
15	3/0/2	4/0/1	4/0/1	3/0/?		
20	4/0/1	5/0/0	4/0/1	4/0/1	5/0/0	3/0/2
25	0/0/5	0/0/5	0/0/5	0/0/5		
30	0/0/5	0/0/5	0/0/5	0/0/5	0/0/5	0/0/5
60	0/0/5		0/0/5		0/0/5	0/0/5

Numbers in brackets indicate which day after inoculation the parasites were harvested
Figures indicate the number of mice died/infected/not infected (determined by the dye test)
RT means room temperature

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DEOXYRIBONUCLEIC ACID RELATEDNESS AMONG SPECIES OF SLOWLY-GROWING MYCOBACTERIA

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Baess I Deoxyribonucleic acid relatedness among species of slowly growing mycobacteria Acta path microbiol scand Sect B 87 221-226 1979

DNA DNA hybridization is a reliable method for determining the phylogenetic relationship between bacterial strains The hybridization kinetics for DNA from different slowly growing mycobacteria were measured optically in a spectrophotometer The results indicate that *M tuberculosis* and *M bovis* belong to one species *M avium* and *M intracellulare* are two species but some serotypes now designated *M intracellulare* actually belong to *M avium*

Key words Nucleic acid hybridization mycobacterium classification

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Much work has been carried out during the past 25 years with the aim of classifying mycobacteria Doubts and divergent opinions still remain concerning the species status of a number of strains Measurement directly on genetic material is the most reliable method for determination of bacterial relationships A bacterial genome consists of thousands of genes and therefore testing of a small number of criteria may not result in adequate determination of the relatedness of the genomes Personal interpretation of results and varying views as to how much weight should be ascribed to the individual characteristic may add to the difficulty of [illegible]

[illegible]

between them at the subspecies level

Results [illegible]

[illegible] present paper reports [illegible] with DNA from slowly growing mycobacteria in particular from *M tuberculosis* and *M bovis* and from *M avium* and *M intracellulare* The significance of the homology percentages found is elucidated by means of hybridization experiments at varying temperatures and the taxonomic consequences are discussed

MATERIAL AND METHODS

Bacterial strains The strains used are shown in Tables 1 and 2

Isolation and purification of DNA The method has been described previously (3) Some of the strains with particularly smooth colonies could not be disrupted in the pressure cell press However if they were washed with isopropylalcohol after harvesting they could be handled in the usual way

Deoxyribonucleic acid hybridization The DNA was used at a concentration of 40 µg/ml in 3 SSC (0 45 M sodium chloride 0 045 M trisodium citrate pH 7) and 25% formamide The hybridizations were follow[illegible]

[illegible] taxonomy selection was made of three pairs of strains with a high a

DISCUSSION

The present investigation of *Toxoplasma gondii* confirms the results obtained by *Jacobs et al* (1960) with respect to the different labilities of endozoites and cystozoites in artificial gastric juice As shown in Table 2, cystozoites were infective for mice after incubation in pepsin-HCl solution for at least three hours whereas an equal number of strain RH endozoites (Table 1) were completely eradicated after incubation under the same conditions for 25 minutes

When the infectivity of *Toxoplasma gondii* after incubation at 37°C is compared with that at room temperature (Table 1) the effect of pepsin which is assumed to be reduced at room temperature, is not detectable Furthermore when comparing the infectivity of parasites incubated in HCl solution with and without pepsin (Tables 1 and 2), the presence of pepsin has no noticeable effect on the destruction process Its effect is probably comparable to that of trypsin and the lability of endozoites should be related to increased permeability rather than to proteolytic susceptibility This was confirmed by the action of methylene blue which diffused faster into endozoites than into cystozoites

Fig 1 shows different rates of destruction for endozoites of strains RH and DUE when incubated in HCl solution Curve B was found to represent the destruction of strain 178 endozoites also since the virulence of that strain is approximately the same as that of strain DUE Thus it seems possible by the action of hydrochloric acid to distinguish between endozoites with respect to their virulence

However the different rate of destruction shown in the figure was too small to account for the differences shown in the two tables This is considered due to the presence of cystozoites among the endozoites harvested from mouse peritoneum cavity after inoculation of avirulent parasites The cystozoites were too few for detection under the microscope though they obviously were present on the sixth and seventh day after inoculation (Table 2) The presence of cystozoites on the fourth day post inoculation when the parasites were only slightly more resistant to hydrochloric acid than virulent ones (Table 1) is possible in view of the results of *Dubey & Frenkel* (1976) that cystozoites are present in mice three days after inoculation of tissue cysts

In the present investigation no cystozoites were found in mouse peritoneum cavity seven days after inoculation of virulent parasites (Table 1) This is in agreement with an earlier report that virulent *Toxoplasma* parasites do not form cysts in mice and rabbits (*Pettersen* 1977) However caused by parasites of the RH strain have been observed in rabbit tissue culture (*Shimada et al* 1974) The *in vitro* formation of RH cysts occurred only in the presence of specific antibodies As there was no indication in the present work that virulent parasites are less antigenic than avirulent ones it is difficult to believe that antibodies should be the stimulus for cyst or cystozoite formation

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TABLE 2 *Homology Percentages between Different Species of Mycobacteria Determined by DNA DNA Hybridization*

Bacterial strain	*M tuberculosis* H37Rv	*M bovis* wild strain	*M tuberculosis* wild strain
M tuberculosis wild strain	99 0 (4 2)		H
M bovis wild strain	102 3 (2 9)	H	
M bovis BCG Copenhagen	100 4 (2 0)	103 0 (1 8)	
M kansasii ATCC 12478	30 7 (3 7)		
M avium serotype 3 wild strain	24 7 (5 6)		
M xenopi SSC 989			19 7 (2 7)

The results are the means of four determinations standard deviation in brackets
H indicates the results for DNAs from the same strain according to this method they are always close to 100%

TABLE 3 *Homology Percentages Determined by DNA DNA Hybridization at Different Temperatures*

Bacterial strains	Tm −15° C	Tm −25° C	Tm −35° C	Δ° C Tm
M bovis + M bovis BCG	103 4	102 9	105 2	0 5
M fortuitum + M farcinogenes var senegalense	28 1	47 2	62 1	1 2
M tuberculosis + M xenopi	−2 1	19 7	35 3	0 9

Tm Midpoint of the thermal denaturation of the DNA in hybridization solutions
Δ° C Tm Difference in Tm measured before and after hybridization at Tm −25° C

T
M
hab
diff

DISCUSSION

By means of the technique used in this study for DNA DNA hybridization among different species we have not observed homology percentages in the interval between 90 and 52 This indicates the reliability and objectivity of the method for determination of the phylogenetic relatedness of mycobacteria at the species level

The fact that the difference in Tm before and after hybridization is not larger regardless of the homology percentage must be because the bonds achieved by this method are relatively heat-stable This is not in accordance with the findings of Brenner *et al* (11) where it would appear that the thermal stability can be correlated with the percentage of unpaired bases This discrepancy is presumably due to the different techniques used The particular composition of salt and formamide in the solution might have some influence (27) The presence of homologous single stranded DNA which forms specific base pairs is probably more important in this connection This aspect needs further elucidation

The results in Table 1 indicate a solution of the taxonomic problems within the so-called *M avium-M intracellulare* complex A homology percentage of 96 indicates strongly that *M intracellulare* Davis serotype 8 belongs to the same species as *M avium* The other strains of *M intracellulare* Boone serotype 12 Howell serotype 14 and Yandle serotype 16 are indistinguishable but they are different from *M avium* Thus the strains examined can be assigned to two species *M avium* including *M avium* serotypes 1, 2 and 3 and *M intracellulare* Davis and *M intracellulare* comprising the three

medium and a low homology percentage, respectively. Hybridizations were made with DNA from these strains at temperatures 10° C higher and 10° C lower than the usual, and the stability of the hybrids formed at temperature Tm −25° C was measured by determination of their Tm values.

RESULTS

No homology percentages have been observed in the interval between 90 and 52. DNAs with a homology percentage of more than 90 are therefore considered as belonging to strains of the same species, and DNAs with a homology percentage below 52 as belonging to strains of different species.

This conclusion is confirmed by the measurements at three different temperatures, the usual Tm −25° C and at a 10° C higher or lower temperature (Table 3). Hybrids with a high homology percentage form more specific base pairs than hybrids with a low homology percentage, as can be seen from the decrease of the homology percentage at the 10° C higher hybridization temperature. This decrease disappears for a homology percentage of 103, but is about 20 % for homology percentages of 47 and 20. More non-specific base pairs seem to be formed at Tm −35° C.

Non-specific base pairing should lower the Tm temperature of the hybrid, but this has not been found in these three cases at hybridization temperature Tm −25° C. This is discussed later.

The results shown in Tables 1 and 2 are mean values of four determinations, the standard deviation (from 1–8 %) is shown in brackets.

The strains of *M. avium* and *M. intracellulare* in Table 1 can be divided clearly into two groups: one consisting of *M. avium* and *M. intracellulare* Davis and the other comprising the other *M. intracellulare* strains. The *M. scrofulaceum* strain is definitely different from both *M. avium* and *M. intracellulare* and *M. xenopi* shows an even smaller relatedness with the other three species.

TABLE 1 *Homology Percentages between Different Species of Mycobacteria Determined by DNA-DNA Hybridization*

Bacterial strain	*M. avium* serotype 2 ATCC 25291	*M. intracellulare* Davis serotype 8 J 1868	*M. intracellulare* Yandle serotype 16 Mark Robert	*M. scrofulaceum* ATCC 19073	*M. xenopi* SSC 989
M. avium serotype 1 ATCC 15769	101.1 (3.2)				
M. avium serotype 2 ATCC 25291	H	89.5 (4.4)			
M. avium serotype 3 wild strain	103.8 (6.2)				
M. intracellulare Davis serotype 8, wild strain	95.9 (2.7)	98.3 (0.6)			
M. intracellulare Howell serotype 12, P 42	51.9 (4.9)	48.6 (4.5)	98.8 (2.6)		
M. intracellulare Boone serotype 14, ATCC 25169	47.8 (6.3)	50.3 (5.1)	95.1 (2.0)	40.4 (7.4)	25.5 (3.2)
M. intracellulare Yandle serotype 16, Mark Robert	51.4 (7.5)	50.5 (4.6)	H		
M. scrofulaceum ATCC 19073	34.4 (7.0)			H	
M. xenopi, SSC 989	24.8 (6.7)			26.6 (3.6)	H

The results are the means of four determinations, *standard deviation in brackets*.
H indicates the results for DNAs from the same strain; according to this method [illegible] close to 100%.

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other serotypes of *M intracellulare* These results support the proposal of *Anz et al* (2) that the strains in the intermediate group of *M intracellulare* actually belong to *M avium* and that two species exist viz *M avium* and *M intracellulare* Such a presumption was put forward already in 1967 by *Runyon* (26)

Bradley & Bond (10) likewise found a homology percentage of 48 between *M avium* and *M intracellulare* *Magnusson* (21) using sensitins could differentiate between *M avium* and *M intracellulare* but not between *M avium* and *M intracellulare* Davis (22) *Anz et al* (1) divided *M avium* and *M intracellulare* into three groups on the basis of serotyping and virulence for hens viz *M avium* with virulence an intermediate group with varying virulence and *M intracellulare* without virulence Using sensitins *Anz et al* (2) could not distinguish the intermediate group to which *M intracellulare* Davis belongs from the *M avium* group but the *M intracellulare* group without virulence could be distinguished from *M avium* Serotypes Boone and Yandle belonged to the *M intracellulare* group In their epidemiological study *Meissner & Anz* (23) found that the majority of strains isolated from human beings with lung affections caused by these mycobacteria consisted of *M avium* and strains belonging to the intermediate group An ecological study revealed that only these strains were found in animals Strains belonging to the *M intracellulare* group were not isolated from living organisms *Kubin et al* (19) found their precipitinogen e only in *M avium* and in *M intracellulare* strains belonging to *Anz et al* s intermediate group *Bennedsen* (6) was not able to distinguish between *M avium* serotype IV and *M intracellulare* Davis by means of the direct flurorescence antibody reaction *Engbæk et al* (12) have reported that *M intracellulare* Davis resembles *M avium* as regards virulence and its ability to grow at high temperatures but that it resembles *M intracellulare* in some other characteristics *Tsukamura & Mizuno* (33 34) using a thin layer chromatography method found that some *M intracellulare* strains resemble *M avium* Using numerical taxonomy distinction between *M avium* and *M intracellulare* has not been possible (31 32) or has been doubtful (17 18 24 35) *Thoen et al* (30) could not find any difference between the strains by means of gas liquid chromatography of their fatty acids

Studies of the DNA base composition of these bacteria offer no solution to the problem since they do not achieve any differentiation (5 28)

The homology percentages shown in Table 2 indicate that a change should be made in the designations of *M tuberculosis* and *M bovis*

The fact that *M tuberculosis* H37Rv has proved to be identical with a wild strain of both *M tuberculosis* and *M bovis* and with *M bovis* BCG is in correlation with *Bradley* s (7 9) results of hybridization with DNAs from the same species This demonstrates clearly that classification of bacteria on the basis of biochemical tests and virulence examinations alone may not reveal their phylogenetic relationship Hybridization at a temperature which should reveal non specific base pairing shows an unchanged homology percentage (Table 3) The base ratios and genome sizes are identical (5) Homology percentages between *M tuberculosis* and *M avium* and *M kansasii* are in good agreement with the findings of *Bradley* (7) and *Gross & Wayne* (14)

M bovis BCG has always been regarded as an avirulent variant of *M bovis* *M bovis* has been known since 1896 (29) when it was designated a bovine tubercle bacillus It has been a subspecies of *M tuberculosis* up to 1957 (25) and has first been validly published as *M bovis* in 1970 (16) It has always been recognized that *M bovis* was different from *M tuberculosis* on the basis of virulence for rabbits and by means of biochemical tests for which reason it has been considered that it should have species status However the indistinguishability of the DNAs suggests that the one species *M tuberculosis* should be re introduced with subspecies designations *M tuberculosis* *M bovis* *M bovis* BCG and perhaps *M africanum* *M tuberculosis* and *M bovis* cannot be distinguished on the basis of their pathogenicity for man or by means of sensitins (20) *Gimpl & Weiszfeiler* (13) and *Jensen et al* (15) found no difference in the antigens of *M tuberculosis* and *M bovis* On the basis of numerical taxonomy they are generally similar (17 31 32) However *Wayne* (35) was able to distinguish them by selecting characters for an Adansonian analysis of taxonomy

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Calculation of the homology percentages was kindly carried out in the Biostatistical Department (Chief *W Weis Bentzon*) of Statens Seruminstitut My best thanks are also due to *B Mansa* Department of Biophysics for performing the molecular weight determinations of the DNA The strain of *M farcinogenes* var *senegalense* was kindly provided by *M Magnusson* of the Tubercul n Department

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NITROBLUE TETRAZOLIUM (NBT) REDUCTION BY BACTERIA

Some Properties of the Reaction and Its Possible Use

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Department of Clinical Bacteriology of the Karolinska Institute at Serafimerlasarettet Stockholm Sweden

Urban T & Jarstrand C Nitroblue tetrazolium (NBT) reduction by bacteria Some properties of the reaction and its possible use Acta path microbiol scand Sect B 87 227–233 1979

All the *S albus E coli* and *P aeruginosa* strains examined reduced nitroblue tetrazolium (NBT) to dark blue formazan The amount of formazan produced was proportional to the number of bacteria Under the same growth conditions an equal number of bacteria of various strains produced different amounts of formazan However there were statistically verified differences in the NBT reduction between the three species examined The NBT reduction took place in all phases of growth but was most intense in the early logarithmic phase NBT was found to be toxic for bacteria and the different strains had varying sensitivity to that effect The NBT reaction was markedly enhanced by phenazine methosulphate (PMS) The blue colour of formazan produced from NBT has an advantage over the red colour from triphenyltetrazolium chloride (TTC) if the reaction occurs in the presence of haemoglobin often present in biological materials With NBT and PMS 10^6–10^7 bacteria are needed to form detectable amounts of formazan The NBT reduction by bacteria may be useful for measuring the influence of bactericidal bacteriostatic or growth stimulating factors on bacteria

Key words Tetrazolium salts bacteria

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Tetrazolium salts especially triphenyltetrazolium chloride (TTC) are used widely as markers of dehydrogenase activity in biological systems TTC is used as neoplasm indicator in selective staining of tumour tissues (2) The TTC reduction of *Mycoplasma pneumoniae* is employed for its identification and for measurement of antibodies against that organism (7 12) Also the capacity of tubercle bacilli to reduce TTC is utilized for determination of the viability of BCG vaccines (15) In a detailed review article *Pergam* (11) described several techniques using TTC for indicating the presence of bacteria or bacterial inhibitors in food and other materials and as a means of selection in bacteriological media Studies on microbiological effects of tetrazolium salts other than TTC such as iodonitrotetrazolium (INT) (4) neotetrazolium chloride (NTC) blue tetrazolium (BT) and nitroblue tetrazolium (NBT) (6 8) have also been reported

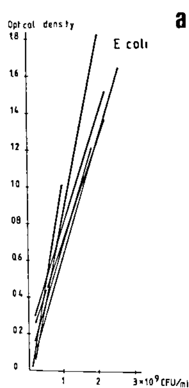
The reduction of almost colourless NBT to dark blue formazan by polymorphonuclear leukocytes is well recognized (9, 10) NBT reduction has been

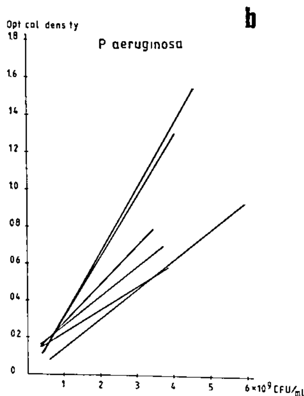
might be useful in bacteriological work Some comparative experiments were also made with TTC, so far the most commonly used tetrazolium salt

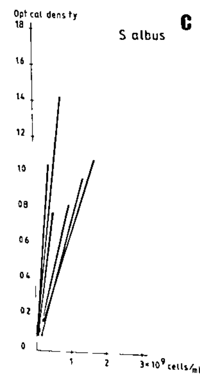
MATERIAL AND METHODS

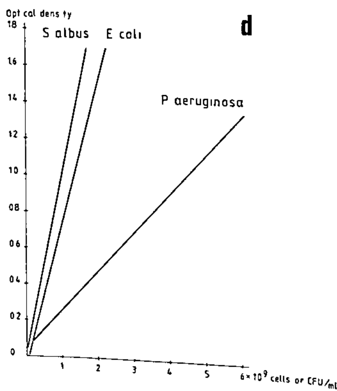
Bacteria

laceum, and *M gordonae* observed by thin-layer chromatography of lipids after uptake of ^{35}S-methionine Kekkaku *53* 85–89, 1978
35 *Wayne L G* Selection of characters for an adansonian analysis of mycobacterial taxonomy J Bact *93* 1382–1391, 1967


a
Optical density
E coli
18
16
14
12
10
08
06
04
02
0
1
2
3×10⁹ CFU/ml


b
Optical density
P aeruginosa
18
16
14
12
10
08
06
04
02
0
1
2
3
4
5
6×10⁹ CFU/ml


c
Optical density
S albus
18
16
14
12
10
08
06
04
02
0
1
2
3×10⁹ cells/ml


d
Optical density
S albus
E coli
P aeruginosa
18
16
14
12
10
08
06
04
02
0
1
2
3
4
5
6×10⁹ cells or CFU/ml

bath at 37°C in order to be in the exponential growth phase when resuspended in fresh nutrient broth for the NBT test The number of colony forming units (CFU) per ml of the suspension was determined by colony counts of serial dilutions in nutrient agar In experiments with staphylococci known to aggregate to CFU of varying sizes the number of bacteria was estimated by counting in a Burker chamber by phase contrast microscopy The numbers of bacteria thus obtained will be referred to as CFU

Procedure for NBT Reduction Test

A 0 1% solution of NBT (BDM Chemical Ltd Poole England) in Hanks solution was used in all experiments Amounts of 0 5–1 ml of bacterial suspension containing from 10^7–10^9 CFU per ml were placed in plastic test tubes (100 × 16 mm) mixed with the same volume of the NBT solution and allowed to react for 30–45 min in a 37°C water bath with a rocking device The reaction was then interrupted by adding the same volume (1 or 2 ml) of 0 5 N HCl The tubes were centrifuged at 1 000 *g* for 15 min and the supernatant was decanted and the pellet extracted with 3 ml dimethylsulphoxide by vigorous shaking Thereafter the optical density (OD) of the extract was determined in a spectrophotometer (Vitatron manual DCP) at 572 nm

The following points were examined

Comparison of the NBT reduction of Different Bacterial Species and Strains

Six strains of *S albus E coli* and *P aeruginosa* were used Nutrient broth was added to 0 1 0 2 0 3 etc up to 1 0 ml of broth cultures containing about 10^9 CFU/ml to a final volume of 1 0 ml 1 ml NBT solution was added to each tube and the mixtures were incubated for 45 min The amounts of formazan were determined as already described

Statistical analysis Regression lines were calculated for the relationships between the number of CFU of each strain on one side and the formazan production (OD value) on the other (Fig 1 a b c) Wilcoxon s rank sum test (13) was used for comparison of the β values representing the slopes of the lines for the different strains Furthermore a line was calculated for each species based on the means of the β values for the individual strains and passing through the grand mean of CFU numbers and OD values (Fig 1 d)

Influence of Different NBT Concentrations on the NBT reduction of an E coli *Strain*

From a culture of one of the *E coli* strains four identical series of 10 tubes were made as described under point 1 To the tubes of each series 1 ml of NBT solution in each of the concentrations 0 1 0 075 0 05 and 0 025% was added (Fig 2) After incubation for 45 min the experiment was completed as described above

NBT reduction by E coli *during Various Phases of Growth*

50 ml of nutrient broth inoculated with *E coli* was incubated at 37°C with agitation for 24 hours Each quarter of the first hour of cultivation each hour up to 8 hours and at 24 hours 0 5 ml aliquots of the culture were withdrawn mixed with 0 5 ml of NBT solution and incubated for 30 min The amounts of formazan produced were measured as described and the numbers of CFU were determined in parallel samples (Fig 3)

Determination of the Toxic Effect of NBT on Bacteria

a) Using three strains of *E coli* two series of eight tubes with 0 5 ml of bacterial suspension were prepared and 0 5 ml NBT solution was added to each tube The numbers of CFU before NBT addition and after incubation for 5 10 15 20 25 30 and 60 min at 37°C were determined for each strain by colony counting from serial dilutions from the tubes in the first series The second series of tubes was used for assay of the amounts of formazan (OD) produced (Fig 4)

b) To 0 5 ml of broth suspensions of 10 strains of *S albus E coli* and *P aeruginosa* 0 5 ml NBT solution was added and the mixture was allowed to react for 15 min at 37°C In this experiment the extent of bacterial survival was measured in a simplified way Serial ten fold dilutions of the reaction mixtures were prepared in nutrient broth and incubated overnight at 37°C The highest dilution showing growth was taken as an expression of the size of the viable population

Comparison of the NBT and TTC reduction by E coli *and* S albus

Experiments were performed in the same way as described under point 1 but with the addition of NBT to one series and TTC to another Since the solubilities of tetrazolium salts are different the highest possible concentrations were used i e 0 2% for TTC and 0 1% for NBT the concentrations in which the salts have generally been used

Effect of Accelerating Agents on Tetrazolium Reduction by E coli

a) To four series each consisting of 0 1 0 2 etc up to 0 5 ml of *E coli* culture nutrient broth was added to a final volume of 0 5 ml Two series received 0 5 ml nutrient broth enriched with glucose giving a final concentration of 0 5% while two other series received 0 5 ml plain broth To each tube in the two series 1 ml of either NBT or TTC solution was added The mixtures were incubated for 30 min

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Fig 1 a b c Regression lines for the relationships between numbers of CFU (for gram negative rods) or numbers of bacteria (for staphylococci) and the amounts of formazan produced (OD) by six strains of *E coli* (a) *Pseudomonas* (b) and *S albus* (c)

Fig 1 d Regression lines for each of three different species based on the mean of the slopes for the strains passing through the grand mean value of bacterial numbers and NBT reduction

TABLE 1 Bactericidal Effect of NBT on 10 Strains of *Staphylococcus albus* *Escherich a coli* and *Pseudomonas aeruginosa* The Survival is Calculated from the Number of CFU before (a) and after (b) 15 minutes of Incubation with NBT $\frac{b}{a}$

Strain		Number of CFU Before addition of NBT (a)	After 15 min incubation with NBT (b)	Survival $\frac{b}{a}$
S albus	mean	10^{8}	10^{3}	10^{5}
	range	10^{6}–10^{10}	no growth –10^{5}	10^{8}–10^{2}
E coli	mean	10^{9}	10^{6}	10^{3}
	range	10^{8}–10^{10}	10^{4}–10^{7}	10^{5}–10^{2}
P aerug nosa	mean	10^{8}	10^{7}	10^{1}
	range	10^{8}	10^{6}–10^{8}	10^{2}–10^{0}

Determination of the Toxic Effect of NBT on Bacteria

a) In Fig 4 the mean numbers of CFU and the average amounts of formazan produced are shown for three *E coli* strains before the addition of NBT and after 5 10 15 20 25 30 and 60 min A decrease in the number of CFU was observed during the first 15 min after which the numbers were quite constant NBT reduction continued at about the same rate throughout the whole observa tion period

b) The numbers of living bacteria before and after incubation for 15 min with NBT and the survival defined as the number of living bacteria after 15 min divided by the initial number will be seen from Table 1 *S albus* strains were most sensitive to the toxic action of NBT and their survival displayed the greatest variation *E coli* strains were on an average 100 times less sensitive than *S albus* and the variation was less while *P aeruginosa* strains were the most resistent and showed even less variation

Comparison of the NBT and TTC reduction by E coli *and* S albus

Under the same experimental conditions the correlation between the OD values and the numbers of bacteria was good in both species for both TTC (r 0 93 0 97) and NBT (r 0 99) For *E coli* the optical densities of eluated red formazan were higher (β 1 88) than those for blue formazan (β 0 66) However *S albus* was much more efficient in reducing of NBT than of TTC (β 1 16 0 51)

Effect of Accelerating Agents on Tetrazolium Reduc tion by E coli

The reduction of NBT by *E coli* was not affected by the addition of 0 5% glucose to the plain broth of the reaction mixture while under the same conditions the reduction of TTC was found to be increased 1 4 to 3 5 times dependent on the number of bacteria

As shown in Table 2 PMS addition potentiated the NBT reduction by *E coli* The most intense colour development was obtained with the highest concentration of PMS tested (0 004 mg/ml) This concentration gave a more than five fold increase of the OD value after incubation for 15 min During further incubation it was still increased but to a lower degree When an analogous experiment with TTC was performed no acceleration of the reduc tion was observed even with a 10 times higher PMS *concentration* Spontaneous colour develop ment was not observed in control tubes containing sterile broth with the same concentration of PMS but without NBT or TTC

In order to ascertain whether the NBT reduction of *E coli* was proportional to the number of reacting cells also when the reaction was accelerated by PMS varying numbers of bacteria were allowed

TABLE 2 Accelerating Effect of Phenazine Methosul phate on NBT reduction by *Escherichia coli*

Concentration of PMS in mg per ml of reaction micture	Formazan production (OD) Time of incubation (min) 15	30	45
0 004	0 703	0 757	0 903
0 002	0 353	0 476	0 525
0 001	0 280	0 368	0 374
0	0 130	0 199	0 210

b) To 0 5 ml of *E coli* culture containing about 10^9 CFU/ml 0 5 ml of either NBT or TTC solution with phenazine methosulphate (PMS) was added After preparatory experiments different concentrations of PMS were chosen for NBT and TTC For TTC concentrations not giving spontaneous colour development were chosen while for NBT the concentrations were those which gave a convenient speed of reaction with the number of bacteria used and which gave results suitable for our recording system Three concentrations of PMS were tested with TTC 0 015 0 03 and 0 06 mg/ml and three with NBT 0 001 0 002 and 0 004 mg/ml The mixtures were incubated for 15 30 or 45 min and the OD s obtained with PMS were compared with those obtained without PMS

c) The correlation between the number of bacteria and the NBT reduction when potentiated with an optimal concentration of PMS was tested A series of tubes was prepared from an *E coli* culture as described under point 1 1 ml of NBT solution with PMS added to give a final concentration of 0 002 mg per ml was then added and the tubes were incubated for 15 min

RESULTS

Comparison of the NBT-reduction of Different Bacterial Species and Strains

When six bacterial strains of *S albus* *E coli* and *P aeruginosa* were cultivated under the same conditions the relationships between the numbers of CFU and the optical densities recorded were quite constant for each strain (correlation coefficients r 0 97–0 99) (Fig 1 a b and c) The β values representing the ability to produce formazan varied considerably between strains of the same species For the *S albus* strains they were from 0 77 to 5 46 The range for *E coli* and *P aeruginosa* strains was narrower viz 0 61–1 26 and 0 16–0 35 respectively The β values for the species differed significantly between *S albus* and *E coli* strains (P $<$ 0 05) between *S albus* and *P aeruginosa* (P $<$ 0 01) and between *E coli* and *P aeruginosa* (P $<$ 0 01) Fig 1 d illustrates further the relationship between the number of bacteria and the formazan production within each of the three species

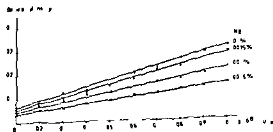


Fig 2 Effect of different NBT-concentrations on the production of formazan by an *E coli* strain

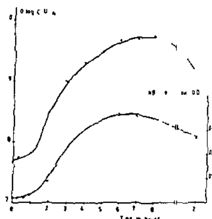


Fig 3 NBT reduction by an *E coli* strain in different growth phases

As will be seen from the regression lines in Fig 1 as many as 10^8 bacteria per ml were needed before formazan could be produced in amounts detectable by our procedure

NBT reduction of E coli *at Different Concentrations of NBT*

Fig 2 shows that with the same number of bacteria a higher formazan production was obtained with a higher concentration of NBT This was the case at all levels of bacterial numbers

NBT reduction by E coli during Various Phases of Growth

The ability of bacteria to reduce NBT in each growth phase (Fig 3) is expressed as

$$\frac{\text{OD formazan}}{\text{number of bacteria}} \times 10^{10}$$

This was 5 2–3 1 for the first 2 hours 2 6–1 3 for the 3rd–5th hour and 0 8–0 6 for the 6th–24th hour Thus the capacity of bacteria to reduce NBT is highest in the early logarithmic phase of growth

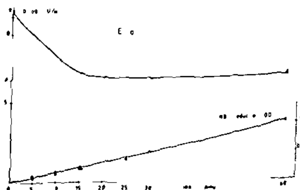


Fig 4 Toxic effect of 0 1% NBT solution after different times of incubation on *E coli* and the NBT reduction

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Determination of the Toxic Effect of NBT on Bacteria

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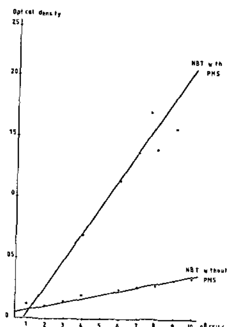


Fig 5 Accelerating effect of phenazine methosulphate (0 002 mg/ml) on the NBT-reduction by *E coli* after incubation for 15 min

to reduce NBT in the presence of optimal PMS concentration A rectilinear relationship (r 0 97) was obtained (Fig 5)

DISCUSSION

In polymorphonuclear leukocytes, the NBT-reduction takes place in the cell membrane and is mediated by membrane-bound oxidase (3, 5) Since the enzymes mediating electron transport and oxidative phosphorylation in bacteria are also localized in the cell membrane, and since NBT competes with oxygen in receiving hydrogen ions from NADPH or NADH (1), the NBT-reduction of bacteria is also likely to occur in the cell membrane For the reaction to occur inside the cell membrane, the latter must probably be damaged by the NBT, since it is supposed normally to be impermeable to that compound

Differences in NBT reduction of various bacterial species and strains can be explained by variations in cell wall structure which might allow different amounts of NBT to reach or penetrate the cell membrane, rather than by differences in oxidative metabolism of the strains Also the differences between various bacteria as regards their sensitivity to the toxic effect of NBT may be due to differences between cell wall structures It would seem that the β-values, representing the NBT reduction abilities, are correlated with the sensitivity of the strains to the toxic action of NBT Perhaps the cell walls of relatively sensitive *S albus* strains are more easily penetrated by NBT than those of resistant Pseudomonas strains

As regards the toxic effect of NBT, it was also noted that the numbers of CFU of three *E coli* strains decreased considerably during incubation with NBT for 15 min However, Fig 4 shows that in spite of this decrease, formazan production continued at about the same rate during the whole observation period Presumably, bacteria which have lost their ability to multiply and thus to form colonies are still able to produce formazan This assumption was corroborated by our own observation (unpublished data) that bacteria treated with γ irradiation loose their colony-forming ability but retain part of their formazan production

It was observed that, in contrast to that formed by leukocytes, formazan produced by bacteria is very rapidly extracted with dimethylsulphoxide even without heating An explanation may be that due to their small size, bacteria form smaller aggregates of formazan, and that these are more easily available for extraction

Only the NBT-reduction of three species was examined, but as virtually all bacteria can reduce TTC (11), they probably also reduce NBT The interaction between bacteria and NBT with different types and numbers of bacteria and different concentrations of NBT, was investigated more closely in order to determine the usefulness of the reaction in different situations Since with PMS stimulation, only 10^6–10^7 bacteria were needed to produce detectable amounts of formazan, the reaction may be employed for rapid detection of bacteria Furthermore, the reaction may be useful for measuring the influence of bactericidal, bacteriostatic or growth-stimulating factors on bacteria An example of this is a method developed for a rapid assay of antibiotics (14)

To summarize our observations on NBT-reduction by bacteria, we would point out some advantages of this reaction in preference to the TTC-reduction, so far the most common tetrazolium compound used Unlike reduction of TTC by *E coli* the reduction of NBT can be accelerated by PMS, an intermediate electron carrier This phenomenon may permit a considerable increase of the sensitivity of the reaction The increase is far more pronounced and more rapidly observed than that caused by glucose on the TTC-reduction Thus the increase is five-fold for NBT with PMS within 15 min, as against two-fold for TTC with glucose after 30 min The strains of staphylococcus tested reduced NBT more rapidly than TTC, which may be useful for the detection of such bacteria The blue

colour of the formazan produced from NBT can be an advantage over the red colour resulting from TTC when the reaction occurs in the presence of haemoglobin for example in blood cultures and in serum or urine samples with haemolysed erythrocytes

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Acta path microbiol scand Sect B 87 235-241 1979

RESISTANCE TYPES IN *ESCHERICHIA COLI*

I Occurrence and Resistance to Ampicillin Carbenicillin and Cephalothin

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Søgaard P Resistance types in *Escherichia coli* I Occurrence and resistance to ampicillin carbenicillin and cephalothin Acta path microbiol scand Sect B 87 235-241 1979

An investigation of the resistance types in lactose fermenting *E coli* is presented The frequency and sensitivity to β lactam antibiotics of different resistance types was investigated The strains were divided into three groups according to sensitivity to penicillin derivatives Group 1 contained the ampicillin carbenicillin sensitive (A s/Ca s) group 2 the ampicillin resistant-carbenicillin sensitive (A r/Ca s) and group 3 the ampicillin-carbenicillin resistant (A r/Ca r) A s/Ca r were not observed One third of the A r strains were sensitive to carbenicillin The distribution of resistance types was different in the three groups Group 1 was dominated by the usual sensitive *E coli* Group 3 contained a very high proportion of multiresistant types The IC_{50} against ampicillin carbenicillin and cephalothin of 55 strains was determined Group 3 (A r/Ca r 25 strains) was much more ampicillin resistant than group 2 (A r/Ca s 16 strains) Group 2 was less sensitive to carbenicillin than group 1 (A s/Ca s 14 strains) Group 3 did not differ significantly from group 1 with respect to cephalothin sensitivity while group 2 was much more resistant than the others

Key words *Escherichia coli* resistance types β lactam antibiotics

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Most strains of *E coli* are sensitive to ampicillin (A) and carbenicillin (Ca) Some of the A resistant (A r) strains are sensitive to Ca (Ca s) *Korner* (1976) found 78 8% of 580 *E coli* strains in a hospital material sensitive to A (A-s) but 92 1% were Ca-s

The occurrence of multiresistance in gram negative enteric bacteria has been described in a paper by *Bergfors et al* (1972) who observed that 27% of 460

from

three

multi

in hospital specimens Among these 25 the most frequent patterns of resistance traits (resistance types) were A Ce Su S (Ce = cephalothin Su = sulphonamide S = strep…

Infections with A r *E coli* occur very often If some of these could be treated with Ca or Ce more toxic antibiotics could be avoided

The intention of the present investigation was to clarify the occurrence of resistance types in *E coli* isolated mainly from a hospital material The resistance against A and Ca has been correlated to resistance to different antibiotics The sensitivity to A Ca and Ce has been investigated for the different resistance types Besides the epidemiological knowledge the investigation also gives information about the use of Ca and Ce in the treatment of infections with A r *E coli*

MATERIALS AND METHODS

A Routine Methods

The information that initiated this investigation was obtained from the routine registration of microbiological specimens at Statens Seruminstitut Regional Laboratory, Odense University Hospital, Odense, Denmark This laboratory receives *urine, pus, sputum, blood and* spinal fluid for bacterial examination from Funen County, the main source being Odense University Hospital The *Enterobacteriaceae* were isolated on Conradi-Drigalski plates and the lactose fermenting organisms were identified by the following reactions mobility and oxygen requirement in semi solid agar, Voges-Proskauer reaction, indole formation, acid and gas production in glucose broth, decarboxylation of lysine and ornithine, fermentation of malonate, liquefying of gelatin and H_2S formation

The strains were tested for antibiotic sensitivity by the direct agar diffusion method The medium antibiotic discs and the method used are described in the instruction book from *A/S Rosco* (1978–79) The medium, Danish Blood Agar, was obtained from Statens Seruminstitut, Copenhagen The composition is described in *A/S Rosco* (1978–79) Strains with an inhibition zone larger than 22 *mm (+ +) were considered sensitive* to the antibiotic in *question* The specimens were routinely tested against nine antibiotics Su P, T S C, E, A, Ca and Ni (P = *penicillin low C* = *chloramphenicol* E = erythromycin and Ni = nitrofurantoin)

The *resistance types (resistance to Su T, S and C)* of 500 consecutive lactose fermenting *E coli* strains were *recorded from routine registration in the period 27 iv 77* to 21 v 77 Each patient contributed only once The strains were divided *into three groups Group I* contained the A-s/Ca-s bacteria group 2 the A r/Ca s and group 3 the A-r/Ca-r No A-s/Ca r strains were observed

The 500 strains comprised 75 A r (groups 2 and 3) To get a greater number of strains in these groups the resistance types of 76 additional (151 total) consecutive A-r strains were recorded (until 16 vi 77)

The information thus obtained was used to elaborate *Tables 2 and 3*

From April 77 to January 78 55 lactose fermenting *E coli* strains were collected from the routine specimens Only the five most prevalent resistance types in group 3 (see Table 2) and the types SuTS, SuS and O (O = no resistance to Su, T, S or C) in groups 1 and 2 were collected The period of sampling the strains was longer than the period *of recording the 151 A-r strains in order* to obtain at least 4 of the less frequent types in each subgroup

B Additional Investigations

The 55 selected *strains (Table 1) were rechecked* with regard to sensitivity pattern and biochemical reactions These latter included beside the eleven reactions mentioned above the following nitrate reduction urease production fermentation of mannitol, sorbitol sucrose rhamnose, trehalose, inositol and production of arginine dihydrolase *All 55 strains were confirmed to be* lactose fermenting *E coli* All were facultatively anaerobic reduced nitrate, were Voges-Proskauer negative produced indole, acid and gas in glucose and mannitol fermented sorbitol (with one exception) and trehalose None produced urease, fermented inositol or malonate liquefied gelatin or produced H_2S All except three could decarboxylate lysine

The sensitivity of the selected strains was tested against A (Doktacillin®, Astra) Ca (Fugacillin®, Astra) and Ce (Cefalotin®, Glaxo) using the tube dilution *method described by Siboni (1976) Inoculum was* 1μl of an overnight broth culture diluted 10^{-2} containing approx $10^{4\ 4}$ *CFU/ml The control strain included in each* experiment was *E coli* K 12 7458/41 Mean $\log_2 IC_{50}$ of *this strain* was 2 50 (5 7μg/ml) s d 0 20 $\log_2$ n = 13 against A Mean $\log_2 IC_{50}$ against Ca was 3 30 (9 8μg/ml) s d 0 62 $\log_2$ n = 15 Mean $\log_2 IC_{50}$ against Ce was 2 27 (4 8 μg/ml) s d 0 33 $\log_2$ n = 13

TABLE 1 *Distribution of Selected Strains Listed According to Resistance Type Number of Strains*

Resistance type	Group 1 A s/Ca s	Group 2 A r/Ca s	Group 3 A-r/Ca-r	Total
SuTSC			4	4
SuTS	4	4	5	13
Su SC			4	4
Su S	4	4	4	12
O	6	8	8	22
Total	14	16	25	55

Su = sulphonamide, T = tetracycline S = streptomycin C = chloramphenicol O = no resistance to Su T S or C
A-s = sensitive to ampicillin, A-r = resistant to ampicillin Ca s = sensitive to carbenicillin Ca r = resistant to carbenicillin
The strains were used for control of routine biochemical diagnosis and determination of IC_{50}

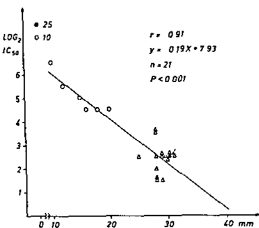


Fig 1 Regression curve for ampicillin showing rela tionship between $\log_2$ IC_{50} and diameter of inhibition zone △ − A s/Ca s strains (14 strains) ○ − A r/Ca s (16 strains among which 10 had inhibition zone 0 mm as marked at the top of the figure) ● = A r/Ca r strains (all 25 strains had inhibition zone 0 mm as marked on the figure) ▲ = control strain (also marked by an arrow)

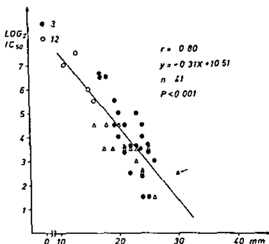


Fig 3 Regression curve for cephalothin 12 A r/Ca s and 3 A r/Ca r strains had inhibition zone 0 mm For list of signs see legend to Fig 1

For the strains tested the diameter of the inhibition zone on 14 cm blood plates was measured The discs used were a set of A Ca and Ce (Neo sensitabs® A/S Rosco DK 2630 Taastrup Denmark) The inoculum was 0 3 ml of an overnight infusion broth culture diluted 10^{-4} The inoculum was spread with a sterile bent glass rod and incubated for 18h at 35°C before reading

Since the selection of the strains was based on sensitivity testing by the direct agar diffusion method the relationship to the tube dilution test is illustrated The regression curve of A is shown in Fig 1 A diameter greater than 22 mm corresponds to $\log_2$ IC_{50} less than 3 71 (13 1 µg/ml) The regression curve is shown for Ca in Fig 2 A diameter greater than 22 mm corresponds to log IC_{50} less than 4 88 (29 4 µg/ml) The regression curve of Ce is shown in Fig 3 A diameter greater than 22 mm corresponds to $\log_2$ IC_{50} less than 3 76 (13 6 µg/ml)

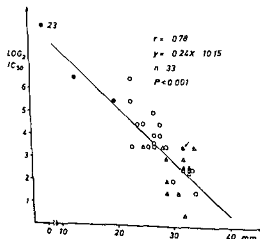


Fig 2 Regression curve for carbenicillin 23 A r/Ca r strains had inhibition zone 0 mm For list of signs see legend to Fig 1

The regression lines shown are not identical with those published by *A/S Rosco* (1978) In this paper the diameter of the inhibition zone is correlated to IC_{50} determined by the tube dilution method while the curve of Rosco is based on the plate dilution method For the same diameter the MIC determined by the first method is about 4 times higher than the corresponding MIC read on the regression line of Rosco

RESULTS

A Frequency of Resistance Types and Traits (Agar Diffusion Method)

The 500 consecutive *E coli* strains recorded from routine registration were distributed as follows group 1 425 group 2 23 group 3 52 strains

Group 1 comprises 85% and the distribution of resistance types in this group is shown in Table 2 (column one) By far the most common type is the usual sensitive *E coli* Resistance type SuS Su T and SuTS are the next most frequent (the last two being equally common)

The 151 consecutive A r strains (groups 2 and 3)

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The sensitivity of the selected strains was tested against A (Doktacillin® Astra) Ca (Fugacillin® Astra) and Ce (Cefalotin® Glaxo) using the tube dilution method described by *Sibon* (1976) Inoculum was 1µl of an overnight broth culture diluted 10^{-2} containing approx $10^{4.4}$ CFU/ml The control strain included in each experiment was *E coli* K 12 7458/41 Mean $\log_2 IC_{50}$ of this strain was 2 50 (5 7µg/ml) s d 0 20 $\log_2$ n = 13 against A Mean $\log_2 IC_{50}$ against Ca was 3 30 (9 8µg/ml) s d 0 62 $\log_2$ n = 15 Mean $\log_2 IC_{50}$ against Ce was 2 27 (4 8 µg/ml) s d 0 33 $\log_2$ n = 13

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Su S	4	4	4	12
O	6	8	8	22
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Su = sulphonamide T = tetracycline S = streptomycin C = chloramphenicol O = no resistance to Su T S or C A s = sensitive to ampicillin A r = resistant to ampicillin Ca s = sensitive to carbenicillin Ca r = resistant to carbenicillin

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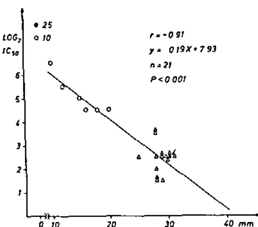


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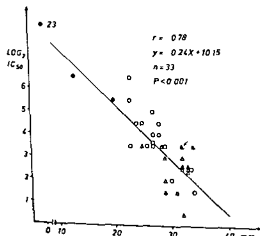


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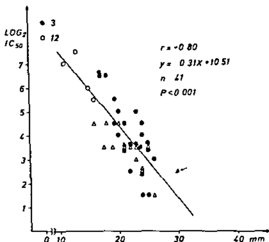


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RESULTS

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The 500 consecutive *E coli* strains recorded from routine registration were distributed as follows group 1 425, group 2 23 group 3 52 strains

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The 151 consecutive A r strains (groups 2 and 3)

TABLE 2 *Percentage of Strains in Groups 1, 2 and 3 According to Resistance Type*

Resistance type	Group 1 A-s/Ca-s	Group 2 A-r/Ca-s	Group 3 A r/Ca r
SuTSC	0 5	2 0	39 0
TSC			1 0
SuTS	4 7	5 9	11 0
TS	2 4	2 0	3 0
SuT C			3 0
T C			
Su SC	0 2	2 0	8 0
SC			1 0
SuT	1 4		2 0
T	4 7	3 9	2 0
Su S	9 9	33 3	8 0
S	1 6	2 0	1 0
Su C			
C			
Su	5 7	11 8	2 0
O	68 9	37 2	19 0
Total	100 0	100 1	100 0

Group 1 numbers are based on 425 strains, group 2 on 51 and group 3 on 100 strains The 151 A-r strains include 51 Ca s (33 8%)

are shown in Table 2 (column two and three) In group 2, in order of decreasing frequency, the most common resistance types are O, SuS, Su SuTS (same order as in group 1) and in group 3 SuTSC, O, SuTS, SuS and SuSC (the last two equally common) The percentage of Ca-s strains among the A-r is 33 8% SuTSC is found in 39 cases out of 100 in group 3 (39%) but only in 1 case out of 51 in group 2 (2%) This resistance type is represented by 2 (0 5%) in group 1 (Table 2) A comparison of the distribution of type SuTSC in group 1 (2 out of 425 strains) with group 3 (39 out of 100) using the exact test of *Fisher* (1950) gives p < 0 001

SuTS is found in 11%, 6% and 5% of groups 3, 2 and 1, respectively (Table 2) SuTC is found only in group 3 (3%) SuSC is found in 8% 2% and 0% of groups 3 2 and 1 respectively The triple resistance types SuTS, SuTC and SuSC are more common in group 3 than in groups 2 and 1 A comparison of the frequency of these types in group 3 (22 out of 100 strains) with group 1 (21 out of 425) by the exact test of *Fisher* (1950) gives p < 0 001

The resistance type SuS is represented with 8%, 33% and 10%, and type O with 19%, 37% and 69% in groups 3, 2 and 1, respectively (Table 2)

The percentage of resistant strains to different antibiotics among the 500 *E coli* strains is shown in Table 3 The results originate from the routine registration (see Materials and Methods)

B Sensitivity to β-Lactam Antibiotics (Tube Dilution Method)

The sensitivity of 55 strains to A, Ca and Ce is shown in Table 4

Ampicillin If every subgroup (= resistance type) is compared with the other subgroups in the same group no significant differences are found by Wilcoxon's test (W), with p > 0 05 except in group 3 between subgroup SuSC and subgroup O, where 0 05 > p > 0 02 W is a weak test, especially when $n_1 = n_2 = 4$ but in support of the conclusion is that the range in subgroups is almost identical (within the same group) The mean $\log_2$ IC_{50} in groups 1, 2 and 3 is 2 39 6 34 and ≥ 10 12 (5 2, 80 9 and ≥ 1112 μg/ml) respectively (subgroup SuSC is excluded in group 3)

Group 3 is more resistant than group 2 (W, p < 0 01) Subgroup SuSC in group 3 is excluded, but this subgroup is even more resistant than the others See also Fig 1

Carbenicillin No significant differences are found between subgroups within each group using W (p > 0 05) The range in subgroups is almost identical The group mean $\log_2$ IC_{50} is 2 46, 3 88 and ≥ 11 62 (5 5, 14 7 and ≥ 3145 μg/ml)

TABLE 3 *Percentage of* E coli *Strains Resistant to Different Antibiotics*

Antibiotic	Su	T	S	C	A	Ca	Ni
500 consecutive strains	29 4	17 2	26 2	6 0	15 0	10 4	5 4[a]
Group 1 (A s/Ca s) 425 strains	22 3	13 4	19 3	0 7	0	0	–
Group 2 (A r/Ca s) 51 strains	55	14	47	4	100	0	–
Group 3 (A r/Ca r) 100 strains	73	61	72	52	100	100	–

Direct agar diffusion method Routine registration
Ni = nitrofurantoin
[a] Only 446 strains

TABLE 4 *Sensitivity of 55 Strains to Ampicillin Carbenicillin and Cephalothin in Groups 1 2 and 3 According to Resistance Type* $Log_2 IC_{50}$ *(24h) Is Shown*

Resistance type	Group 1 A s/Ca s			Group 2 A r/Ca s			Group 3 A r/Ca r		
Antibiotic	A	Ca	Ce	A	Ca	Ce	A	Ca	Ce
SuTSC							≥12 5	≥12 5	6 5
							11 5	≥12 5	3 5
							9 5	≥12 5	3 5
							9 0	12 0	4 5
SuTS	1 5	0 5	3 5	4 5	2 0	7 5	12 0	≥12 5	5 0
	2 5	2 0	3 5	7 5	4 5	10 5	9 0	11 5	3 5
	2 5	3 0	4 5	6 5	3 5	8 5	12 0	≥12 5	6 5
	1 5	3 5	1 5	6 5	4 5	8 5	10 5	≥12 5	4 0
SuSC							9 5	6 5	≥10 5+
							10 0	≥12 5	2 5
							≥12 5	≥12 5	6 5
							≥12 5	≥12 5	6 5
							11 5	11 5	3 5
SuS	2 5	3 5	4 5	5 5	3 5	7 5	11 5	≥12 5	5 5
	2 5	1 5	3 5	8 5	6 5	9 5	11 0	≥12 5	1 5
	1 5	1 5	3 5	6 5	3 5	9 0	7 5	5 5	≥10 5+
	3 5	3 5	3 5	6 5	3 5	9 5	10 0	11 5	4 0
O	2 5	3 0	3 0	6 5	4 0	9 0	10 5	12 0	4 5
	2 0	2 5	2 5	5 0	2 5	7 0	9 5	12 0	4 5
	3 5	2 5	4 5	4 5	1 5	6 0	9 5	11 0	2 5
	2 5	2 5	3 5	6 5	4 0	≥10 5	11 5	≥12 5	5 0
	2 5	2 5	2 5	6 5	4 5	10 5	8 0	12 0	1 5
	2 5	2 5	3 5	4 5	3 5	5 5	9 0	≥12 5	3 5
				8 5	5 5	11 5	9 5	11 5	3 5
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Group mean	2 39	2 46	3 39	6 34	3 88	≥ 8 72	≥10 12[a]	≥11 62	≥ 4 64

[a] subgroup SuSC excluded
Each triplet represents one strain

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Ni nitrofurantoin
[a] Only 446 strains

TABLE 4 *Sensitivity of 55 Strains to Ampicillin Carbenicillin and Cephalothin in Groups 1 2 and 3 According to Resistance Type Log₂ IC₅₀(24h) Is Shown*

Resistance type	Group 1 A s/Ca s			Group 2 A r/Ca s			Group 3 A r/Ca r		
Antibiotic	A	Ca	Ce	A	Ca	Ce	A	Ca	Ce
SuTSC							≥12 5	≥12 5	6 5
							11 5	≥12 5	3 5
							9 5	≥12 5	3 5
							9 0	12 0	4 5
SuTS	1 5	0 5	3 5	4 5	2 0	7 5	12 0	≥12 5	5 0
	2 5	2 0	3 5	7 5	4 5	10 5	9 0	11 5	3 5
	2 5	3 0	4 5	6 5	3 5	8 5	12 0	≥12 5	6 5
	1 5	3 5	1 5	6 5	4 5	8 5	10 5	≥12 5	4 0
							9 5	6 5	≥10 5+
SuSC							10 0	≥12 5	2 5
							≥12 5	≥12 5	6 5
							≥12 5	≥12 5	6 5
							11 5	11 5	3 5
SuS	2 5	3 5	4 5	5 5	3 5	7 5	11 5	≥12 5	5 5
	2 5	1 5	3 5	8 5	6 5	9 5	11 0	≥12 5	1 5
	1 5	1 5	3 5	6 5	3 5	9 0	7 5	5 5	≥10 5+
	3 5	3 5	3 5	6 5	3 5	9 5	10 0	11 5	4 0
O	2 5	3 0	3 0	6 5	4 0	9 0	10 5	12 0	4 5
	2 0	2 5	2 5	5 0	2 5	7 0	9 5	12 0	4 5
	3 5	2 5	4 5	4 5	1 5	6 0	9 5	11 0	2 5
	2 5	2 5	3 5	6 5	4 0	≥10 5	11 5	≥12 5	5 0
	2 5	2 5	2 5	6 5	4 5	10 5	8 0	12 0	1 5
	2 5	2 5	3 5	4 5	3 5	5 5	9 0	≥12 5	3 5
				8 5	5 5	11 5	9 5	11 5	3 5
				7 5	5 0	9 0	9 5	11 0	3 0
Group mean	2 39	2 46	3 39	6 34	3 88	≥ 8 72	≥10 12[a]	≥11 62	≥ 4 64

[a] subgroup SuSC excluded
Each triplet represents one strain

TABLE 2 *Percentage of Strains in Groups 1, 2 and 3 According to Resistance Type*

Resistance type	Group 1 A-s/Ca s	Group 2 A-r/Ca-s	Group 3 A r/Ca r
SuTSC	0 5	2 0	39 0
TSC			1 0
SuTS	4 7	5 9	11 0
TS	2 4	2 0	3 0
SuT C			3 0
T C			
Su SC	0 2	2 0	8 0
SC			1 0
SuT	1 4		2 0
T	4 7	3 9	2 0
Su S	9 9	33 3	8 0
S	1 6	2 0	1 0
Su C			
C			
Su	5 7	11 8	2 0
O	68 9	37 2	19 0
Total	*100 0*	100 1	100 0

Group 1 numbers are based on 425 strains, group 2 on 51 and group 3 on 100 strains The 151 A-r strains include 51 Ca s (33 8%)

are shown in Table 2 (column two and three) In group 2, in order of decreasing frequency, the most common resistance types are O, SuS, Su, SuTS (same order as in group 1) and in group 3 SuTSC, O, SuTS, SuS and SuSC (the last two equally common) *The percentage of Ca-s strains among the A-r is 33 8%* SuTSC is found in 39 cases out of 100 in group 3 (39%) but only in 1 case out of 51 in group 2 (2%) *This resistance type is represented* by 2 (0 5%) in group 1 (Table 2) A comparison of the distribution of type SuTSC in group 1 (2 out of 425 strains) with group 3 (39 out of 100) using the exact test of *Fisher* (1950) gives $p < 0\ 001$

[illegible]

2 [illegible]

in group [illegible]

0% *of groups 3, 2 and 1 respectively The triple* resistance types SuTS, SuTC and SuSC are more common in group 3 than in groups 2 and 1 A *comparison of the* frequency of these types in group 3 (22 out of 100 strains) with group 1 (21 out of 425) by the exact test of *Fisher (1950)* gives $p < 0\ 001$

The resistance type SuS is represented with 8%, 33% and *10%*, and type O with 19%, 37% and 69% *in groups* 3, 2 and 1, respectively (Table 2)

The percentage of resistant strains to different antibiotics among the 500 *E coli* strains is shown in Table 3 The results *originate* from the routi registration (see Materials and Methods)

B Sensitivity to β-Lactam Antibiotics (Tube Diluti Method)

The sensitivity of 55 strains to A, Ca and Ce shown in Table 4

Ampicillin If every subgroup (= resistance ty *is compared with the other subgroups* in the san group no significant differences are found l Wilcoxon's test (W) with $p > 0\ 05$ except in gro 3 between subgroup SuSC and subgroup O, whe $0\ 05 > p > 0\ 02$ W is a weak test, especial when $n_1 = n_2 = 4$ but in support of tl conclusion is that the range in subgroups is almo identical (within the same group) The mean lo IC_{50} *in groups* 1 2 and 3 is 2 39, 6 34 and ⩾ 10 1 (5 2, 80 9 and ⩾ 1112 μg/ml) respective (subgroup SuSC is excluded in group 3)

Group 3 is more resistant than group 2 (W, p 0 01) Subgroup SuSC in group 3 is excluded, bu *this subgroup is even more resistant than the other* See also Fig 1

Carbenicillin No significant differences are foun between subgroups within each group using W (> 0 05) The range in subgroups is almo identical *The group mean* log_2 IC_{50} *is 2 46, 3 8* and ⩾11 62 (5 5, 14 7 and ⩾3145 μg/ml)

TABLE 3 *Percentage of E coli Strains Resistant to Different Antibiotics*

Antibiotic	Su	T	S	C	A	Ca	Ni
500 consecutive strains	29 4	17 2	26 2	6 0	15 0	10 4	5 4[a]
Group 1 (A s/Ca s) 425 strains	22 3	13 4	19 3	0 7	0	0	–
Group 2 (A r/Ca s) 51 strains	55	14	47	4	100	0	–
Group 3 (A r/Ca r) 100 strains	73	61	72	52	100	100	–

Direct agar diffusion method Routine registration
Ni – nitrofurantoin
[a] Only 446 strains

TABLE 4 *Sensitivity of 55 Strains to Ampicillin Carbenicillin and Cephalothin in Groups 1 2 and 3 According to Resistance Type Log2 IC50 (24h) Is Shown*

Resistance type	Group 1 A s/Ca s			Group 2 A r/Ca s			Group 3 A r/Ca r		
Antibiotic	A	Ca	Ce	A	Ca	Ce	A	Ca	Ce
SuTSC							≥12 5	≥12 5	6 5
							11 5	≥12 5	3 5
							9 5	≥12 5	3 5
							9 0	12 0	4 5
SuTS	1 5	0 5	3 5	4 5	2 0	7 5	12 0	≥12 5	5 0
	2 5	2 0	3 5	7 5	4 5	10 5	9 0	11 5	3 5
	2 5	3 0	4 5	6 5	3 5	8 5	12 0	≥12 5	6 5
	1 5	3 5	1 5	6 5	4 5	8 5	10 5	≥12 5	4 0
SuSC							9 5	6 5	≥10 5+
							10 0	≥12 5	2 5
							≥12 5	≥12 5	6 5
							≥12 5	≥12 5	6 5
SuS	2 5	3 5	4 5	5 5	3 5	7 5	11 5	11 5	3 5
	2 5	1 5	3 5	8 5	6 5	9 5	11 5	≥12 5	5 5
	1 5	1 5	3 5	6 5	3 5	9 0	11 0	≥12 5	1 5
O	3 5	3 5	3 5	6 5	3 5	9 5	7 5	5 5	≥10 5+
	2 5	3 0	3 0	6 5	4 0	9 0	10 0	11 5	4 0
	2 0	2 5	2 5	5 0	2 5	7 0	10 5	12 0	4 5
	3 5	2 5	4 5	4 5	1 5	6 0	9 5	12 0	4 5
	2 5	2 5	3 5	6 5	4 0	≥10 5	9 5	11 0	2 5
	2 5	2 5	2 5	6 5	4 5	10 5	11 5	≥12 5	5 0
	2 5	2 5	3 5	4 5	3 5	5 5	8 0	12 0	1 5
				8 5	5 5	11 5	9 0	≥12 5	3 5
				7 5	5 0	9 0	9 5	11 5	3 5
							9 5	11 0	3 0
Group mean	2 39	2 46	3 39	6 34	3 88	≥ 8 72	≥10 12[a]	≥11 62	≥ 4 64

[a] subgroup SuSC excluded
Each triplet represents one strain

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Resistance type	Group 1 A-s/Ca-s	Group 2 A-r/Ca s	Group 3 A r/Ca r
SuTSC	0 5	2 0	39 0
TSC			1 0
SuTS	4 7	5 9	11 0
TS	2 4	2 0	3 0
SuT C			3 0
T C			
Su SC	0 2	2 0	8 0
SC			1 0
SuT	1 4		2 0
T	4 7	3 9	2 0
Su S	9 9	33 3	8 0
S	1 6	2 0	1 0
Su C			
C			
Su	5 7	11 8	2 0
O	68 9	37 2	19 0
Total	100 0	100 1	100 0

Group 1 numbers are based on 425 strains, group 2 on 51 and group 3 on 100 strains The 151 A-r strains include 51 Ca-s (33 8%)

are shown in Table 2 (column two and three) In group 2, in order of decreasing frequency, the most common resistance types are O, SuS, Su, SuTS (same order as in group 1) and in group 3 SuTSC, O, SuTS, SuS and SuSC (the last two equally common) The percentage of Ca-s strains among the A-r is 33 8% SuTSC is found in 39 cases out of 100 in group 3 (39%) but only in 1 case out of 51 in group 2 (2%) This resistance type is represented by 2 (0 5%) in group 1 (Table 2) A comparison of the distribution of type SuTSC in group 1 (2 out of 425 strains) with group 3 (39 out of 100) using the exact test of *Fisher* (1950) gives $p < 0\ 001$

SuTS is found in 11%, 6% and 5% of groups 3, 2 and 1, respectively (Table 2) SuTC is found only in group 3 (3%) SuSC is found in 8%, 2% and 0% of groups 3, 2 and 1 respectively The triple resistance types SuTS, SuTC and SuSC are more common in group 3 than in groups 2 and 1 A comparison of the frequency of these types in group 3 (22 out of 100 strains) with group 1 (21 out of 425) by the exact test of *Fisher* (1950) gives $p < 0\ 001$

The resistance type SuS is represented with 8%, 33% and 10%, and type O with 19%, 37% and 69% in groups 3, 2 and 1, respectively (Table 2)

The percentage of resistant strains to different antibiotics among the 500 *E coli* strains is shown in Table 3 The results originate from the routine registration (see Materials and Methods)

B Sensitivity to β-Lactam Antibiotics (Tube Dilution Method)

The sensitivity of 55 strains to A, Ca and Ce is shown in Table 4

Ampicillin If every subgroup (= resistance type) is compared with the other subgroups in the same group no significant differences are found by Wilcoxon s test (W), with $p > 0\ 05$ except in group 3 between subgroup SuSC and subgroup O, where $0\ 05 > p > 0\ 02$ W is a weak test especially when $n_1 = n_2 \approx 4$ but in support of the conclusion is that the range in subgroups is almost identical (within the same group) The mean $\log_2$ IC_{50} in groups 1 2 and 3 is 2 39 6 34 and ≥ 10 12 (5 2, 80 9 and ≥ 1112 μg/ml) respectively (subgroup SuSC is excluded in group 3)

Group 3 is more resistant than group 2 (W, $p < 0\ 01$) Subgroup SuSC in group 3 is excluded but this subgroup is even more resistant than the others See also Fig 1

Carbenicillin No significant differences are found between subgroups within each group using W ($p > 0\ 05$) The range in subgroups is almost identical The group mean $\log_2$ IC_{50} is 2 46, 3 88 and ≥11 62 (5 5, 14

described in this paper These can be divided into two groups (Table 4) One group (marked O on the figures) is resistant to A sensitive to Ca and highly resistant to Ce (Fig 3) and the other group (marked ●) is highly resistant to A and Ca (Fig 2) but more sensitive to Ce than the first group (Fig 3) The two groups do not differ with respect to biochemical reactions and further investigations are necessary to elucidate the difference

I am grateful to Cand Stat *Susanne Møller* Biostatistical Department Statens Seruminstitut for advice on the statistical part of the work and to Dr *K Siboni* for encouragement and inspiration

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Group 2 is more resistant than group 1 ($p < 0\ 01$ by W) See Fig 2 But both are to be classified as Ca s according to the clinical evaluation

Cephalothin No significant differences are found between subgroups within each group (W, $p > 0\ 05$) Range in subgroups is almost identical The mean $\log_2 IC_{50}$ of groups 1, 2 and 3 are 3 39, $\geqslant$8 72 and $\geqslant$4 64 (10 5, $\geqslant$421 and $\geqslant$24 9 μg/ml)

Group 2 is more resistant than group 1 and group 3 (W, $p < 0\ 01$ in both cases) *No significant difference is found between group 1 and group 3* (W, $0\ 10 > p > 0\ 05$) *Correspondingly Fig 3 shows* that group 1 and group 3 are intermingled while group 2 is placed in the upper part of the graph There seems to be a biological dividing line at 16–17 mm inhibition zone between groups 1 and 3 on the one hand and group 2 on the other Groups 1 and 3 thus appear to belong to the same population with respect to cephalothin sensitivity

C *Biochemical Reactions*

No correlation between resistance type and biochemical reactions could be established

DISCUSSION

The percentages of strains resistant to different antibiotics (Table 3) agree very well with the results published by *Korner* (1976) The greatest difference is found with A (15% in this investigation versus 21 2% in that of *Korner*) Otherwise the two sets of percentages are within 2 5% Consequently there is no great difference between the sensitivity of *E coli* in the two areas

Korner (1976) found the most frequent multiresistance type in *E coli* to be SuSACe and SuTSCe In the present paper the most frequent multiresistance types are SuTS SuTSCACa and SuSA C *sensitivity is not recorded in Korner s paper and Ce* sensitivity is not recorded routinely in this laboratory *Bergfors et al* (1972) reported the most frequent multiresistance types in gram negative enteric bacteria to be TSA SuTS and SuTSA

These observations show that the resistance combinations SuS and SuTS are common in *E coli* and suggest that plasmids with at least these resistance traits are of widespread occurrence

If this suggestion is correct, then the combination of Su S and Su T- S should occur more frequent than by chance If the resistance traits were ... of Su and S ... 262 = 0 077 ... consecutively recorded strains this combination actually occurs in 111 strains (0 222) which gives $p < 0\ 001$ by the χ^2 test ($\chi^2 = 151\ 8$ df = 1) The combination SuTS should occur in 0 077 × 0 172 = 0 0132 but the observed number of strains with this pattern is 46 of 500 (0 092) ($p < 0\ 001$ $\chi^2 = 220\ 4$ df = 1) SuTSC would be expected in 0 0132 × 0 060 = 0 0008 of the strains The observed number with this combination is 21 out of 500 (0 042)

It can be concluded that a coupling exists between Su T and S resistance As mentioned in Results there is also a coupling with resistance to penicillin derivatives In the A r/Ca r group the frequency of *multiresistance (SuTSC SuTS SuTC* and *SuSC) is* much greater than in the A s/Ca s group

E coli has been recorded as sensitive to Ca in this investigation if the diameter of the inhibition zone is greater than 22 mm corresponding to $\log_2 IC_{50}$ less than 4 88 ($IC_{50} <$ 29 4 μg/ml) The concentration of Ca in urine after a dose of 1 g Ca i m (normal renal function) is > 1000 μg/ml (*Hewitt & Winters* 1973)

The A r/Ca s *E coli* strains in this material have a mean $\log_2 IC_{50}$ of 3 88 (range 1 5 to 6 5) = 14 7 μg/ml (range 2 83 to 90 μg/ml) It seems reasonable that urinary tract infections with these bacteria can be treated with Ca as the concentration of Ca obtainable in urine is more than 3–5 times the MIC ($IC_{50} \times \sqrt{2}$)

E coli strains resistant to A and sensitive to Ca (group 2) are resistant to Ce (Table 4 and Fig 3) A dose of 1 g Ce i m gives a maximum concentration in urine of 2200–5200 μg/ml (normal renal function) (*Walter & Heilmeyer* 1969) According to Table 4 group 3 (A r/Ca r) has a mean $\log_2 IC_{50}$ of $\geqslant$4 64 (range 1 5 to $\geqslant$10 5) = >24 9 μg/ml (range 2 8 to $\geqslant$1450 μg/ml) Two strains (marked +) have $\log_2 IC_{50} \geqslant 10\ 5$ If these are excluded the mean $\log_2 IC_{50}$ is 4 13 (17 5 μg/ml) It seems probable that urinary tract infections caused by these bacteria will often respond to Ce

Two strains in Table 4 group 3 are marked by + These strains have the same profile as the bacteria in group 2 (*i e* less resistant to Ca than to A and very resistant to Ce) They are included in group 3 because they had an inhibition zone less than 23 mm with the Ca disc They seem to be high variants of group 2 If they are included in group 2 and the 18 values of Ca sensitivity are forced into a normal curve and tested for goodness of fit by the χ^2 test this gives $0\ 70 > p > 0\ 50$ ($\chi^2 = 0\ 863$ 5 classes df *= 2) (Croxton 1959)* This means that *they could* belong to group 2

In the genus *Citrobacter* the species *Citrobacter freundii* usually is A r/Ca s/Ce r *Citrobacter koseri* is A r/Ca r/Ce s (*Southern & Bagby* 1977)

This relationship is similar to the A r *E coli*

Acta path microbiol scand Sect B 87 243–246 1979

OCHRATOXIN A CONTAMINATION OF FOODSTUFFS IN AN AREA WITH BALKAN (ENDEMIC) NEPHROPATHY

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Pavlovic, M Plestina R & Krogh P Ochratoxin A contamination of foodstuffs in an area with Balkan (endemic) nephropathy Acta path microbiol scand Sect B 87 243–246 1979

Ochratoxin A is a nephrotoxic fungal metabolite (mycotoxin) occurring in foodstuffs The compound is causally associated with mycotoxic porcine nephropathy a disease comparable with a human kidney desease Balkan (endemic) nephropathy A survey of 768 samples of foodstuffs (cereals and bread) locally produced in an area of Yugoslavia where Balkan (endemic) nephropathy is prevalent has revealed that ochratoxin A is constantly present in parts of foodstuffs The mean freqency of ochratoxin A contamination of cereals in the study period was 8 7 per cent but a pronounced annual variation was encountered with frequencies of contamination up to 43 per cent These contamination frequencies are higher than those reported elsewhere for foodstuffs for human consumption Thus further evidence is provided to support the hypothesis that ochratoxin A might be a disease determinant of Balkan (endemic) nephropathy

Key words Nephropathy Balkan endemic ochratoxin A foodstuffs

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Balkan (Endemic) Nephropathy (EN) first recognized in the 1950 s is a fatal chronic kidney disease affecting inhabitants of rural areas of Bulgaria Romania and Yugoslavia This disease entity has been the subject of several international meetings the latest being the 3rd Symposium on Endemic Nephropathy in Nis Yugoslavia 1975 (*Strahinjić et al* 1977) EN is characterized by contracted kidneys with changes exclusively in the renal cortex including tubular degeneration interstitial fibrosis and hyalinization of glomeruli (*Heptinstall* 1974) Renal function tests point to the proximal tubules as the primary target site (*Dochev* 1973) Numerous etiological investigations have been carried out covering infectious agents (bacteria virus) genetic factors and trace elements However the etiology of EN remains unknown (*Puchlev* 1974)

Mycotoxic nephropathy is a naturally occurring disease in pigs and poultry Ochratoxin A a dihydro-isocoumarin derivative linked through its 7-carboxy group to L β phenylalanine is a nephrotoxic secondary metabolite (mycotoxin) produced by several species belonging to the fungal genera *Aspergillus* and *Penicillium* (*Krogh* 1976) This compound is a major disease determinant of porcine and possibly also avian nephropathy (*Krogh* 1978) Striking similarities have been observed between EN and ochratoxin A induced porcine nephropathy in terms of changes of renal structure and function thus suggesting common causal relationships (*Krogh* 1974) In order to elucidate the possibility of ochratoxin A as a disease determinant of EN a

from this area (*Krogh et al* 1977)

MATERIALS AND METHODS

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MATERIALS AND METHODS

Selection of geographical area for study The village of Kaniza near Slavonski Brod Croatia Yugoslavia was selected as the study area The prevalence of EN in

Kaniža is well documented, since an epidemiological study conducted in 1974 revealed that 7.3 per cent of the population were affected by EN, and 4.3 per cent were suspected of suffering from the disease (*Hrabar et al* 1976). This village is situated in lowland near the river Sava, a tributary of the Danube. For comparison purposes, the inclusion in the study of nearby areas where EN is supposed not to occur was considered. However, this idea was dropped, since no epidemiological data were available to exclude convincingly the presence of EN in these areas.

Sampling of foodstuffs. Cereal samples (200–500 g) were collected during the autumn and winter from stores in individual homes. Maize and wheat were sampled every year in the period 1972–1976, barley only during 1974–1976. Samples of wheat bread (1975 and 1976) covered to varying extents with colonies of mould were collected from the kitchens of the homes.

Method of analysis for ochratoxin. After grinding 50 g samples were subjected to qualitative thin layer chromatographic analysis (*Nesheim et al* 1973), which included treatment of the TLC plate with ammonia fumes (*Trenk & Chu* 1971); the detection limit was 5 µg/kg. Positive samples were confirmed by the ester formation (*Nesheim et al* 1973) or by *in vitro* transformation of ochratoxin A into ochratoxin α (*Galtier & Alvinerie* 1976). Quantitative

TABLE 1. *Frequency of Ochratoxin A Contamination in Foodstuffs*

Year of Harvest	Number of Samples Analysed	Number of Samples Contaminated	Per Cent Contaminated
Maize			
1972	33	5	15.2
1973	58	5	8.6
1974	272	25	9.2
1975	172	10	5.8
1976	7	0	0
Total	542	45	
Mean			8.3
Wheat			
1972	4	1	25.0
1973	4	0	0
1974	71	6	8.5
1975	36	1	2.8
1976	15	3	20.0
Total	130	11	
Mean			8.5
Wheat Bread			
1975	17	5	29.4
1976	15	1	6.7
Total	32	6	
Mean			18.8
Barley			
1974	14	6	42.9
1975	45	2	4.4
1976	5	0	0
Total	64	8	
Mean			12.5

determination was carried out on samples during the last two years of the study period (covering 30 of the total of 70 samples found positive) according to *Nesheim et al* (1973)

RESULTS

Altogether 768 samples of foodstuffs were analysed 736 samples of cereals and 32 samples of bread

As was to be expected ochratoxin A was the only one of the ochratoxins detected and no ochratoxin B or other metabolites of the ochratoxin group were found The overall frequency of ochratoxin contamination in the cereals was 8 7 per cent but a pronounced variation from year to year was apparent with frequencies ranging from zero to 42 9 per cent (Table 1) In bread the overall frequency of contamination was 18 8 per cent with a pronounced annual variation similar to that found in the cereals

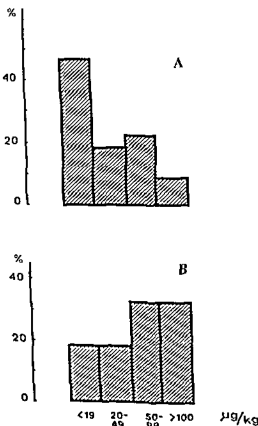


Fig 1 Distribution of levels of ochratoxin A (μg/kg) in contaminated samples of foodstuffs A Maize (21 contaminated samples) B Wheat (6 contaminated samples)

Of the positive samples quantified 10 per cent of the maize samples contained more than 100 μg/kg (Fig 1) whereas 33 per cent of the quantified wheat samples contained levels above 100 μg/kg The highest level encountered was 140 μg/kg In three barley samples the range of concentration was 13 5–25 6 μg/kg

DISCUSSION

The frequencies of ochratoxin contamination in the cereal commodities investigated all of which were locally produced are considerable higher than those reported previously for cereals intended for human consumption generally not exceeding 3 per cent (review *Krogh* 1978) As cooking procedures only result in minor reduction of the ochratoxin content (*Trenk et al* 1971 *Harwig et al* 1974) the population in this endemic area is likely to consume ochratoxin A in prepared food The samples of bread consisted of wheat breads with visible colonies of mould Although mouldy parts of bread would normally be discarded by cutting out the superficial slides with the mould colonies this procedure does not exclude consumption of ochratoxin since the toxin may have penetrated into deeper layers of the bread It is also possible that the toxin content detected was partly or totally the result of primary contamination of the wheat grains from which the breads were produced

In conclusion ochratoxin A has been detected in foodstuffs used by the population in an area where Balkan (endemic) nephropathy is prevalent The frequency of ochratoxin contamination is higher than that reported from other areas of the world for cereals for human consumption Thus further evidence is provided supporting the hypothesis that ochratoxin A might be a disease determinant of Balkan (endemic) nephropathy

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Acta path microbiol scand Sect B 87 247–252 1979

ELECTRON MICROSCOPY OF A FILAMENTOUS, SEGMENTED BACTERIUM ATTACHED TO THE SMALL INTESTINE OF MICE FROM A LABORATORY ANIMAL COLONY IN DENMARK

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FAO/WHO Collaborating Centre for Research and Reference in Toxoplasmosis[1] and Department of Biophysics[2] Statens Seruminstitut Copenhagen Denmark

Ferguson D J P & Birch Andersen A Electron microscopy of a filamentous segmented bacterium attached to the small intestine of mice from a laboratory animal colony in Denmark Acta path microbiol scand Sect B 87 247–252 1979

A filamentous segmented bacterium was observed in the small intestine of the SSC AH stock of mice from the Statens Seruminstitut (Denmark) animal colony but was absent in golden hamsters and guinea pigs from the same colony The bacterium is attached to the epithelial cell by a special segment (holdfast) and causes specific changes in the epithelial cell at the site of attachment Once attached the bacterium appears to undergo a complex life cycle which involves the development of a long filament divided into a number of segments within which holdfasts or spores are formed This organism is morphologically identical to a bacterium found in mice and rats in the USA but this is the first report of such an infection in Europe

Key words Bacterium mice small intestine attachment ultrastructure

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A filamentous segmented bacterium which attaches to the epithelial cells of the small intestine has been observed in a number of strains of mice and rats examined the United States of America (USA) (1 2 3 4 5 10 11 12 13 14) This organism is thought to belong to the *Arthromitaceae* (3) although its exact classification is still unknown The organism does not appear to be pathogenic although the microvilli of the epithelial cells at the point of attachment are affected (5 14) The organism has been examined by both scanning and transmission electron microscopy (1 2 3 4 5 10 11 13 14) Detailed descriptions of the attachment zone and the development within the filament of the bacterium have been [illegible] (2 3 4 14) Recently a morphological study describing a possible complex life cycle for the bacterium has been published (2)

While carrying out a project on the ultrastructure of the protozoan parasite *Toxoplasma gondii* the small intestine of mice from the animal colony of the Statens Seruminstitut (SSI) was examined It was found that the mice had a filamentous segmented bacterium attached to the epithelial cells [illegible]

[illegible] morphologically it is very similar if not identical to the bacterium reported in the USA

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MATERIALS AND METHODS

Six mice (2–3 months old) of the SSC AH stock from the SSI animal colony were anaesthetized and their small intestine exposed The small intestine was opened and immersed in 6% glutaraldehyde in cacodylate buffer Small pieces of intestine were removed and fixed overnight Thereafter the material was processed as described previously (6) In summary the samples were postfixed in osmium tetroxide treated with uranyl acetate and embedded in Vestopal Thin sections were stained with uranyl acetate and lead citrate prior to examination with a Philips EM 200 electron microscope

In addition three golden hamsters and three guinea pigs from the SSI animal colony were examined using the same techniques

RESULTS

Of the 12 animals examined from the SSI animal colony only the mice were observed to possess the filamentous segmented bacterium All six mice were infected although the bacterial density was variable The golden hamsters and guinea pigs were all negative (Table 1)

The bacteria were found firmly attached to the epithelial cells by specialized end segments termed holdfasts (2) This segment has a nipple like protrusion which is embedded in a cone like invagination of the plasmalemma of the epithelial cell (Fig 1) The plasmalemma appears to remain intact and is closely applied to the bacterial cell wall (Fig 2) A few blebs on the plasmalemma could be observed towards the bottom of the invagination (Fig 4) Distinct changes occur in the cytoplasm of the epithelial cell around the attachment site Immediately below the plasmalemma is an osmiophilic zone approximately 35 nm wide This is followed by a zone of intermediate density (Figs 2 3 & 5) In certain micrographs it appeared that these changes may be related to a condensation or contraction of the microfilaments of the terminal web (Figs 4 & 5) The bacterial wall appeared to be of the gram positive type (9) and normally consisted of four layers but the region in contact with the epithelial cell lacked the outer two layers (Fig 2)

A number of the organisms appeared as single rods attached to the epithelial cell (Fig 1) A complex life cycle involving a number of developmental stages has been proposed for a similar bacterium in rats (2) In this paper similar development stages for the organism present in the mice of the SSI animal colony will be illustrated

TABLE 1 *Presence of the Filamentous Segmented Bacterium in the Small Intestine of Rodents from the SSI Animal Colony*

Animals	Strain	Animals possessing the bacterium*
Mice	SSC AH	6/6
Golden Hamsters	SSC GH	0/3
Guinea Pigs	SSC AL	0/3

*Represents the number of animals possessing the bacterium to the number of animals examined

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Figures 1–17 are electron micrographs of a filamentous segmented bacterium present in the lumen of the small intestine of mice

A double bar (=) on a figure represents 1 µm and a single bar (–) 100 nm

The following abbreviations are used throughout C = cortex M = microfilaments I = inner layer of the spore wall MV = microvilli O = outer layer of the spore wall P = plasmalemma and TW = terminal web

Fig 1 A section showing the special attachment zone between a bacterium and an epithelial cell ×45 000

Fig 2 An enlargement of part of Fig 1 showing the variation in structure of the bacterial wall between the area in contact with the epithelial cell and that projecting into the lumen Note the intact plasmalemma and underlying osmiophilic zone of the epithelial cell ×90 000

Fig 3 In this micrograph a segmented filament of the bacterium is shown attached to an epithelial cell ×15 000

Fig 4 A section through the attachment zone showing blebs (arrows) on the plasmalemma of the epithelial cell ×90 000

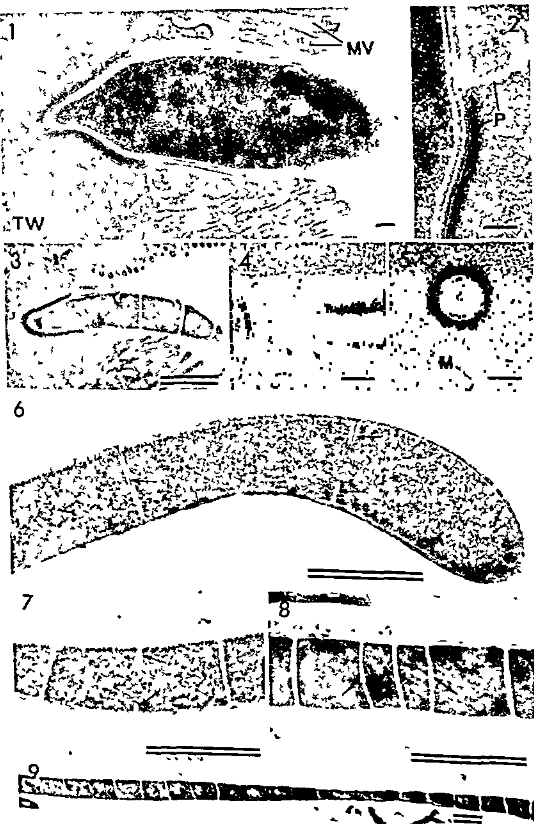
Fig 5 A cross section through the attachment zone Note that the region of intermediate density appears to consist of cytoplasmic microfilaments of the epithelial cell ×90 000

Fig 6 A longitudinal section through part of a bacterial filament showing formation of septa (arrows) to produce primary segments ×30 000

Fig 7 Part of a filament showing the initiation of the septum (arrows) which will divide the primary segment into secondary segments ×30 000

Fig 8 A section through part of a bacterial filament showing two secondary segments which have been divided unequally by transverse septa (arrows) ×30 000

Fig 9 In this section part of a long filament made up of a large number of segments is illustrated × 7 500


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The terminology of *Chase & Erlandsen* (2) will be used to describe the various stages At first the attached rod shaped organism elongates and is divided by transverse septa to form primary segments (Figs 3 & 6) The primary segments are further divided by transverse septa to form secondary segments (Fig 7) At this stage the filaments can be very long and consist of more than 40 segments (Fig 9) A further development of a transverse septum divides the secondary segments unequally (Fig 8) and this septum undergoes rearrangement to form a spherical body (Fig 10) which eventually lies free within the secondary segment or mother cell Then the spherical bodies elongate they achieve a cresentic shape and each divides to form two holdfasts (Figs 11 & 12) At this stage the mother cell may degenerate (Fig 13) and the holdfasts are released to become attached to the epithelium In other cases a spore wall is formed around the two holdfasts This development is initiated by the appearance of a cortex around the daughter organisms (Fig 14) Two layers of the spore coat are then formed around this cortex (Figs 15 16 & 17) This development occurs prior to the degeneration of the mother cell

The initial filament consisting of primary segments is 0 7 µm in diameter and during developments there is a progressive increase in diameter with the filament which contains new holdfasts and spores being 1 5 µm in diameter

DISCUSSION

The ultrastructure of the bacterial cell wall and the changes in the epithelial cell at the site of attachment observed in the present study are similar to those described previously in mice and rats from the USA (2 3 5 10) The possible mechanism of the formation of the attachment has been discussed by *Snellen & Savage* (14) who proposed that the change could be related to a partial phagocytosis of the bacterium which is stabilized by a sol gel transition in the cytoplasm around the attachment site From the present study it would appear that this sol gel transition may be caused by a change in character of the microfilaments of the terminal web as was suggested by *Snellen & Savage* (14)

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Based on a scanning electron microscope study *Blumershine & Savage* (1) suggested that the infection was caused by two types of filamentous bacteria However from the present study and from the results of *Chase & Erlandsen* (2) it seems equally possible that the different diameters observed represent early and late stages of filament development and not two distinct populations

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Fig 10 A section showing the redistribution of the septa (arrows) giving rise to spherical bodies within the secondary segments × 45 000

Fig 11 Part of the filament where the elongated bodies within the secondary segments or mother cells are found in a state of division (arrows) × 45 000

Fig 12 The two holdfasts formed within each mother cell are illustrated in this section × 45 000

Fig 13 A section showing a holdfast within a degenerating mother cell × 45 000

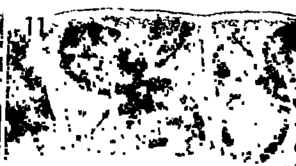
Fig 14 This micrograph shows a very early stage in spore formation The two daughter organisms are surrounded by a cortex on the exterior side of which patches of the dense inner spore wall are present (arrows) × 45 000

Fig 15 This section shows a segment in which the inner layer of the spore wall is almost completely formed × 45 000

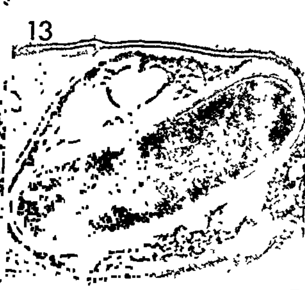
Fig 16 In this part of a filament the segments contain spores in which the inner layer of the spore wall is complete and the outer layer has started to form × 45 000

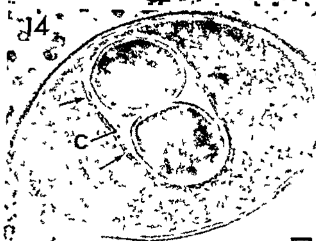
Fig 17 A section through a segment showing a fully formed spore The spore wall consists of a cortex and inner and outer layers × 45 000

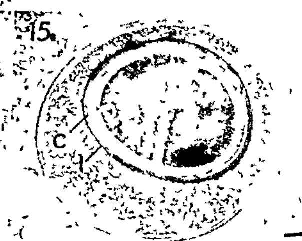


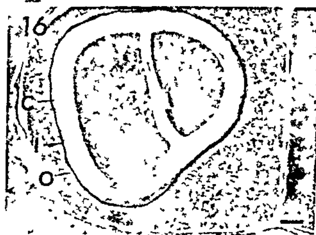

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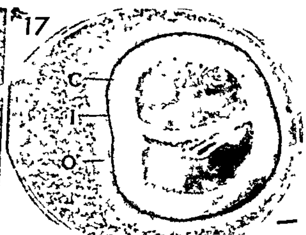

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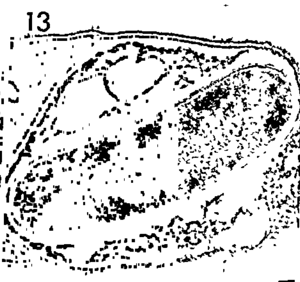
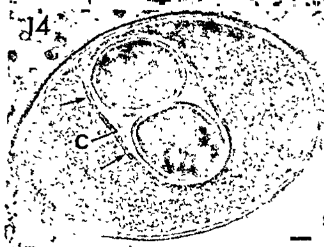
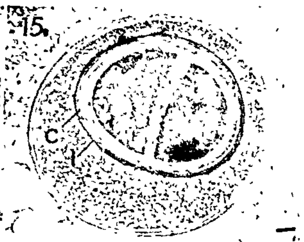
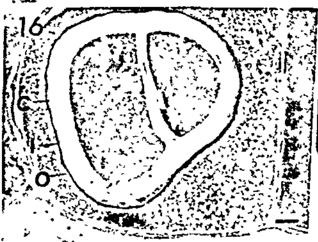
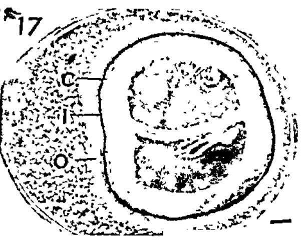
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stock ... mice is a Danish breed of mixed origin but it is possible that this strain initially obtained the

... rodents (golden hamsters and guinea pigs) maintained in the same animal colony were examined These were found to be uninfected It would appear that golden hamsters and guinea pigs

Fig 10 A section showing the redistribution of the septa (arrows) giving rise to spherical bodies within the secondary segments × 45 000

Fig 11 Part of the filament where the elongated bodies within the secondary segments or mother cells are found in a state of division (arrows) × 45 000

Fig 12 The two holdfasts formed within each mother cell are illustrated in this section × 45 000

Fig 13 A section showing a holdfast within a degenerating mother cell × 45 000

Fig 14 This micrograph shows a very early stage in spore formation The two daughter organisms are surrounded by a cortex on the exterior side of which patches of the dense inner spore wall are present (arrows) × 45 000

Fig 15 This section shows a segment in which the inner layer of the spore wall is almost completely formed × 45 000

Fig 16 In this part of a filament the segments contain spores in which the inner layer of the spore wall is complete and the outer layer has started to form × 45 000

Fig 17 A section through a segment showing a fully formed spore The spore wall consists of a cortex and inner and outer layers × 45 000

do not harbour this organism and there are no previous reports of it being present Thus the bacterium seems to be limited to mice and rats An elongated bacterium attached to the ileum of chickens was described by light microscopy (8) but its possible relationship to the bacterium found in the mice and rats is unknown

The bacterium appears to be non-pathogenic to the animals harbouring it but its affect on the physiology of the host is unknown It has been noted that although the bacterium is in contact with the host cells, there is no significant inflammatory response (3, 10, 14) *It is thus possible that the* bacterium is affecting the normal immunological response of the host In addition, since the microvilli are affected at the site of attachment (5, 14) it is possible that heavy infections may affect intestinal function Our findings are reported to draw attention to the fact that experimental mice in Europe like those in the USA can be infected with this bacterium and that such an infection may affect experimental results

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These observations were made during a project on the biology of *Toxoplasma gondii* which was being carried out in co operation with Dr *J Chr Siim* Department of Toxoplasmosis Statens Seruminstitut Copenhagen Denmark and Professor *W M Hutchison* Department of Biology University of Strathclyde Glasgow Scotland The project was supported by grants from the *World Health Organization Geneva the Wellcome Trust* the Danish Medical Research Council and the *W H Ross Foundation*

We are indebted to *K L Fennestad* V M D for supplying animals and to Miss *A G Overgaard* and Mr *F Laursen* for photographic assistance

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ULTRASTRUCTURAL STUDIES ON THE SPORULATION OF OOCYSTS OF *TOXOPLASMA GONDII*

III Formation of the Sporozoites within the Sporocysts

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FAO/WHO Collaborating Centre for Research and Reference in Toxoplasmosis[1] and Department of Biophysics[2] Statens Seruminstitut Copenhagen Denmark and Department of Biology[3] University of Strathclyde Glasgow Scotland

Ferguson D J P Birch Andersen A Siim J Chr & Hutchison W M Ultrastructural studies on the sporulation of oocysts of *Toxoplasma gondii* III Formation of the sporozoites within the sporocysts Acta path microbiol scand Sect B 87 253–260 1979

Sporozoite formation was studied in oocysts which had been allowed to sporulate for 16 24 36 and 48 hours at 27° C The process was initiated within sporocysts which had a fully formed wall A nucleus was situated at each end of the organism Two dense plaques which consisted of two closely applied unit membranes with underlying microtubules were observed close to the limiting membrane in the vicinity of each nucleus At this stage the nuclei possessed an eccentrically located nuclear spindle the poles of which were directed towards the plaques Sporozoite formation continued with a posterior growth of the plaques accompanied by an invagination of the limiting membrane Two sporozoites are formed from each end of the sporocyst As this is occurring the Golgi bodies appear active and numerous membranes and vacuoles are formed Each developing sporozoite encloses a number of these vacuoles (probably precursors of the rhoptries and micronemes) and after the final nuclear division a nucleus is also enclosed The posterior growth of the plaques continues until the formation of sporozoites is completed Thus the mature sporocyst contains four sporozoites and a residual cytoplasmic mass Each sporozoite contains a few polysaccharide granules in addition to the previously mentioned organelles but lacks refractile or crystalloid bodies

Key words *Toxoplasma gondii* sporulation sporozoite formation ultrastructure

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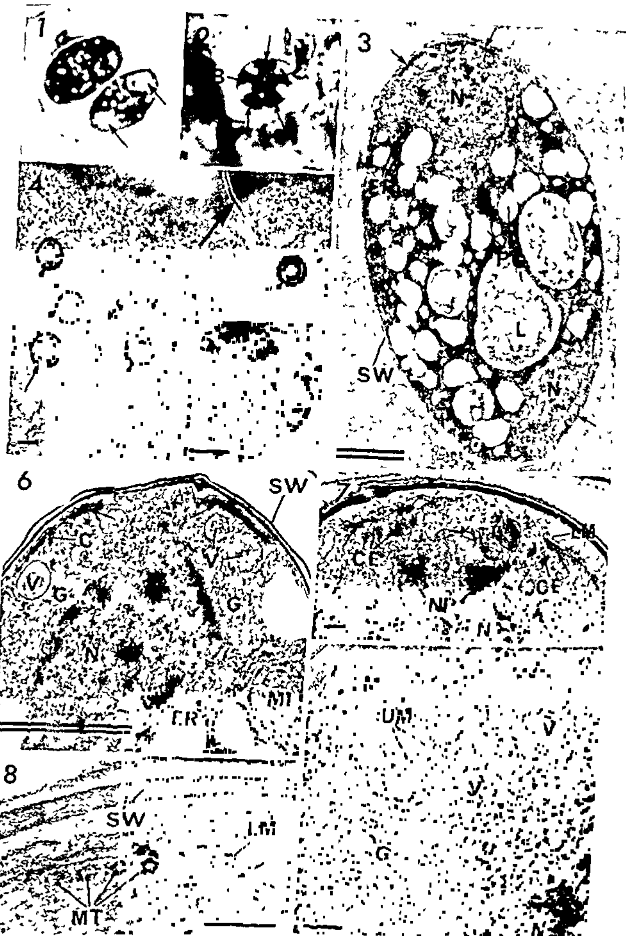
Details of the ultrastructural changes occurring during the sporulation of the oocysts of the coccidian parasite *Toxoplasma gondii* have not been completely described In previous papers we have described the development of the zygote formation and structure of the sporoblasts and formation of the sporocysts (6 7) In this report the formation of the sporozoites within the sporocysts of *T gondii* will be described and compared to that reported for other members of the Sporozoa

MATERIALS AND METHODS

The materials and methods were as described previously (3 6) In this study oocysts which had been allowed to sporulate for 16 24 36 and 48 hours were examined The results are based on the examination of approximately 400 electron micrographs

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Figures 1 and 2 are light micrographs and Figures 3–19 ...

The following abbreviations are used throughout C conoid CE – centriole ER = rough endoplasmic reticulum G = Golgi body L – lipid globule LM = limiting membrane (plasmalemma) MI = mitochondrion MN microneme MP = micropore MT = microtubule N = nucleus NP = nuclear pole NS = nuclear spindle P plaque PG = polysaccharide granule PL pellicle R – rhoptries RB = residual body SP – sporozoite SW – sporocyst wall UM = unit membrane V vacuole (precursor of rhoptry or microneme)

Fig 1 A section of an oocyst showing the two sporocysts which each contains a single cytoplasmic mass In one sporocyst a nucleus can be seen positioned at each end of the organism (arrows) × 2 500

Fig 2 A section through a mature sporocyst within which the sporozoites are visible (arrows) × 2 500

Fig 3 A longitudinal section through an early sporocyst in which a nucleus can be seen at either end of the organism Note the dense plaques in the cytoplasm adjacent to the sporocyst wall (arrows) In addition the cytoplasm contains a number of polysaccharide granules lipid globules and some rough endoplasmic reticulum × 15 000

Fig 4 A tangential section through the limiting membrane of an early sporocyst showing the presence of five micropores (small arrows) Note the junction between two of the plates of the inner layer of the sporocyst wall (large arrow) × 45 000

Fig 5 A section through a multi membranous vacuole observed close to the site of initial sporozoite formation Note that a number of unit membranes are limiting the vacuole (arrow) × 90 000

Fig 6 A section through a sporocyst showing one end of the organism with two dense plaques (arrows) close to the limiting membrane in the region of a nucleus Note the presence of a conoid in the centre of one plaque Golgi bodies a number of vacuoles a mitochondrion and some rough endoplasmic reticulum are also visible × 30 000

Fig 7 A section through one end of a sporocyst showing a nucleus in which a nuclear spindle can be seen Note the centrioles close to the nuclear poles and that these poles are directed towards the two dense plaques (arrows) present close to the limiting membrane of the organism × 45 000

Fig 8 A higher magnification showing the region of a plaque The plaque consists of two closely applied unit membranes (arrows) with underlying microtubules situated adjacent to the limiting membrane of the organism × 160 000

Fig 9 Part of a section in which an extensive Golgi ...

RESULTS

Light Microscopy

At 16 hours sporulation a number of the oocysts possessed two developing sporocysts After 24 hours the majority of oocysts contained two fully formed sporocysts At this stage a few of the sporocysts contained sporozoites At 36 and 48 hours the majority of sporocysts contained fully formed sporozoites

Light microscopy was carried out on 1 µm thick sections stained with toluidine blue Details of the structural changes which occur as the newly formed sporocyst containing a single cytoplasmic mass (Fig 1) develops into a sporocyst containing four sporozoites (Fig 2) could not be resolved using the light microscope

Electron Microscopy

The development of the sporoblast into the sporocyst as well as the formation of the sporocyst wall has been described previously (7)

The newly formed sporocyst is ellipsoidal in shape and contains a single cytoplasmic mass limited by a unit membrane (plasmalemma) external to which is the sporocyst wall The plasmalemma possesses numerous micropores five have been observed in a single thin section (Fig 4) In the cytoplasm a nucleus with associated Golgi bodies is situated at either end of the organism A number of polysaccharide granules lipid globules mitochondria and some strands of rough endoplasmic reticulum are also present (Fig 3)

The first evidence of the initiation of sporozoite formation is the appearance of two dense plaques at either end of the organism close to the limiting membrane and in the vicinity of a nucleus (Figs 3 & 6) The plaques consist of two closely applied unit membranes with underlying microtubules and thus represent the anlagen of the inner membrane complex of the pellicle of the sporozoites (Fig 8) A conoid is present in the central region of the plaques (Fig 6) At this stage an eccentrically located nuclear spindle can be observed within each nucleus (Fig 7) The poles of this spindle are directed towards the developing plaques Centrioles are present close to the nuclear poles (Fig 7) Extensive Golgi bodies are observed close to the nucleus along with numerous membranes and vacuoles of various sizes (Fig 9)

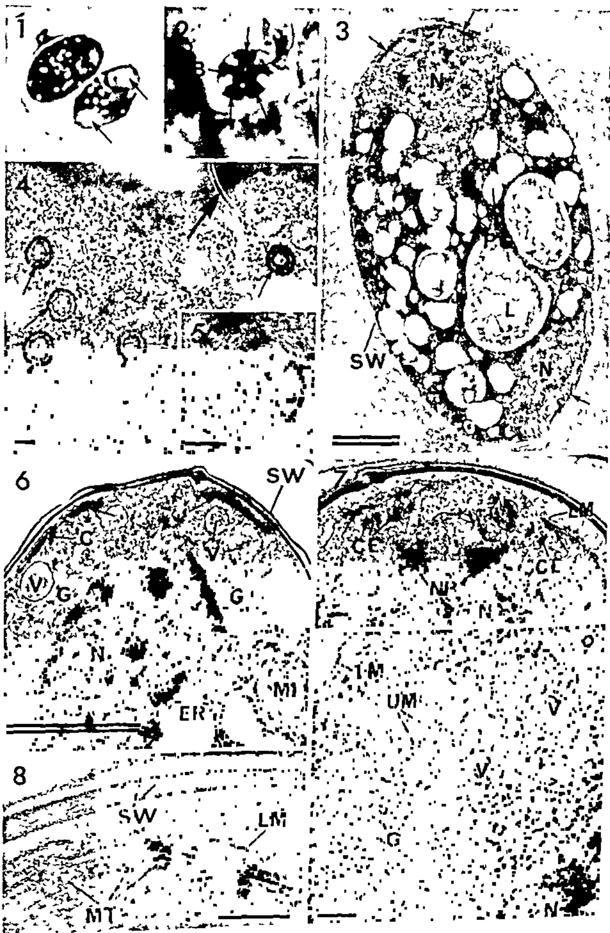

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Fig 10 Part of a section through an early sporocyst showing the posterior growth of a dense plaque (arrows) and the invagination of the limiting membrane × 30 000

Fig 11 A higher magnification of part of Fig 10 showing the invaginated limiting membrane and its close relationship to the dense plaque × 90 000

Fig 12 Part of a section through a sporocyst at a later stage of development than that in Fig 10 In this organism the posterior growth of the sporozoite anlagen has progressed further (arrows) Note that the two poles of the nucleus have moved apart × 30 000

Fig 13 Part of a section through a sporocyst in which the anterior of a sporozoite has been formed by the posterior growth of the plaque (arrows) The cytoplasm of the sporozoite contains a number of vacuoles × 30 000

Fig 14 A higher magnification of part of Fig 13 showing the invaginated plasmalemma and the plaque which consists of two closely applied unit membranes × 90 000

Fig 15 A longitudinal section through a late sporocyst showing three of the developing sporozoites Note that the posterior growth of the plaque (arrows) has enclosed a nucleus in which the nuclear pole can be seen directed towards the anterior of the sporozoite × 15 000

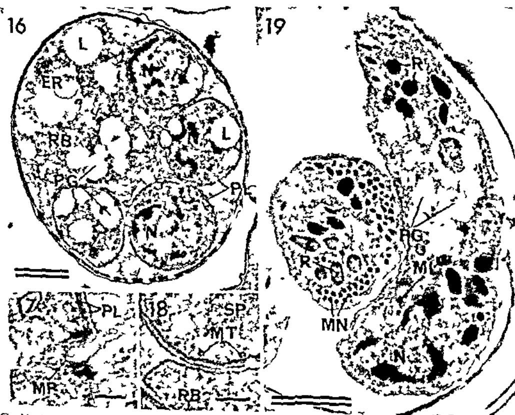


Fig 16 A cross section through a mature sporocyst in which the four sporozoites and the residual body are illustrated × 15 000

Fig 17 Part of a section through a sporozoite showing the periphery with a micropore present on the pellicle × 90 000

Fig 18 Part of a section through a mature sporocyst in which the pellicle of a sporozoite with underlying microtubules can be seen The residual body is limited by a single unit membrane × 90 000

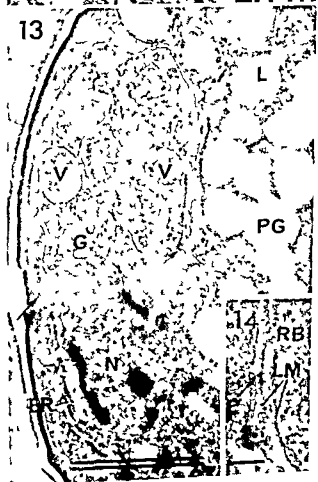
Fig 19 A section presenting part of a sporocyst [illegible]

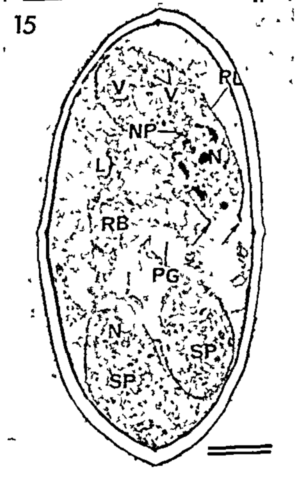
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fractured before the organism was properly fixed and embedded Thus a high percentage of the sporocysts were either not fixed and embedded or destroyed by the cryostat sectioning The formation of the sporocyst wall prior to sporozoite formation is similar to that reported for related families and genera (2 11 14 16 17 18 19) but differs from *E brunetti* (5) where sporocyst wall and sporozoite formation occur simultaneously

In *T gondii* the initiation of sporozoite formation as evidenced by the appearance of dense plaques close to the limiting membrane in the vicinity of the nuclei is similar to that reported for all other members of the Sporozoa in which sporulation has been examined (for references see 5) The process of sporozoite formation occurs by an invagination of the sporozoite anlagen into the cytoplasmic mass of the sporocyst This is similar to that described for *E brunetti* (5) *Coelotropha durchoni* (14) *Diplauxis schreveli* (16) *D hatti* (19) and *Aggregata eberthii* (10 18) As proposed previously (5) this form of development could be related to the fact that the process occurs within the confines of a sporocyst The process observed in *T gondii* is very similar to that described for *E brunetti* (5) except that in *T gondii* two sporozoites are formed from either end of the sporocyst In addition the sporozoite formation in *T gondii* differs from that reported on other members of the Sporozoa in that the final nuclear division occurs during the development of the sporozoites

The majority of studies on asexual multiplication within the Sporozoa have been on merozoite formation (schizogony) although there are a few studies on sporozoite formation (for references see 5) The literature on merozoite formation has been reviewed by *Porchet Hennere* (15) *Hammond* (9) and *Aikawa & Sterling* (1) and from the available information it has been proposed that two types of schizogony can occur In one case the daughter cell formation occurs at the surface of the mother cell while in the other daughter formation takes place within the mother cell (endodyogeny or endopolygony) Except for this aspect both processes are similar and the terms exogenese (exogenesis) and endogenese (endogenesis) respectively have been proposed (24) These terms can also be applied to sporozoite formation From the studies on sporozoite formation of members of Sporozoa it would appear that the organisms undergo exogenesis (for references see5) In the case of *T gondii* however the endogenous forms are characterized as undergoing endogenesis in which the enteric forms multiply by endopolygony (13) and the exoenteric forms by endodyogeny (12) In contrast as the results in the present study show sporozoite formation occurs at the mother cell surface and thus resembles the process of exogenesis Consequently it appears that *T gondii* is the first member of the Sporozoa which can be ultrastructurally shown to undergo both exogenesis and endogenesis depending on the stage of the life cycle examined This emphasizes how closely related the two processes are and although they seem to be characteristic for a particular stage in the life cycle of certain species they should not be used as a species characteristic

A multimembranous vacuole is observed during the early stages of sporozoite formation Its structure is similar to the organelle termed the Golgi adjunct described during endodyogeny of *T gondii* (12) The organelle has been observed during asexual development in both *T gondii* and certain *Eimeria* spp (for references see 4) but its functional significance is still unknown

The sporozoites of *T gondii* possess the conoid rhoptries and micronemes which are characteristic of the infective stages of the coccidia The sporozoites lack however both refractile bodies and crystalloid bodies whereas a few polysaccharide granules are present Thus the sporozoites of *T gondii* differ from those of the closely related *Eimeria* spp (5 20 22 23) which possess refractile bodies and *Isospora canis* (21) which possesses a crystalloid body The function of these bodies is unclear and therefore the significance of their absence in *T gondii* is unknown

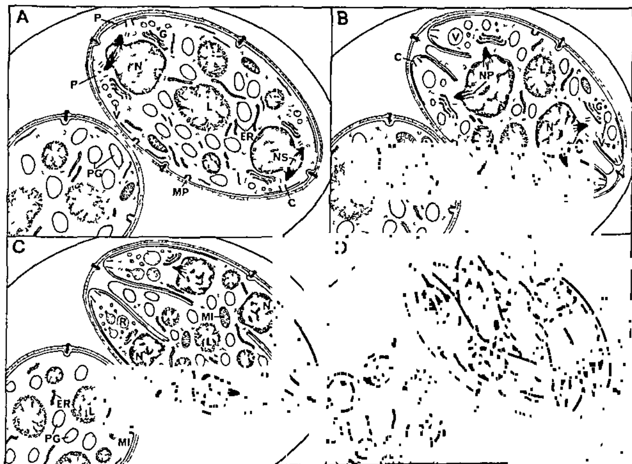
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We are indebted to Mr *J F Dunachie* and Miss *M Reilly* for the maintenance of the SPF cats We gratefully acknowledge Dr *J Blom* for assistance with the light microscopy and Miss *A G Overgaard* and Mr *F Laursen* for photographic assistance

The work was supported by grants from the *World Health Organization* the *Wellcome Trust* the *Danish Medical Research Council* and the *W H Ross Foundation*

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Text Fig 1 A diagrammatical representation of the changes observed during the formation of the four sporozoites (A-C) The structure of the fully sporulated sporocyst is shown in D

Sporozoite formation continues as an inward or posterior growth of the plaques which is accompanied by an invagination of the plasmalemma (Figs 10 & 11) At this time a multi membranous vacuole can be observed close to the plaques (Fig 5) As development proceeds the nuclear poles move apart (Fig 12) and vacuoles with amorphous contents become enclosed by the posterior growth of the plaques (Fig 13) The vacuoles vary in size and probably represent the precursors of the rhoptries and micronemes The plaque can still be seen to consist of two closely applied unit membranes (Fig 14) Two sporozoites are formed from either end of the organism Associated with this development the nucleus present at either end of the organism divides once and the four nuclei thus formed become enclosed within the developing sporozoites (Fig 15) The nuclear pole is retained during this development, and is oriented towards the anterior end of the forming sporozoite (Fig 15) The posterior growth continues and a few mitochondria, polysaccharide granules and some rough endoplasmic reticulum become enclosed by the developing pellicle A posterior pore is formed by the inner layer of the pellicle prior to the release of the fully developed sporozoite from the residual cytoplasmic mass In a few sporozoites lipid globules were also present (Fig 16) Thus the sporocysts at this stage contains four sporozoites plus a residual cytoplasmic mass in which a number of polysaccharide granules, lipid globules, mitochondria and some rough endoplasmic reticulum are present (Fig 16) The residual body is enclosed by a single unit membrane (Fig 18) The sporozoites possess a conoid a nucleus and a number of rhoptries, micronemes polysaccharide granules mitochondria and a few strands of rough endoplasmic reticulum (Fig 19) Each sporozoite is enveloped by a pellicle on which a micropore is present (Fig 17) A diagrammatical representation of the process of sporozoite formation is given in Text Fig 1

DISCUSSION

This study was complicated by the fact that the sporocyst wall is formed prior to the initiation of sporozoite formation This meant that not only the oocyst wall but also the sporocyst wall had to be

Acta path microbiol scand Sect B 87 261–263 1979

BRIEF REPORT

ELISA FOR HERPES SIMPLEX VIRUS (HSV) TYPE SPECIFIC ANTIBODIES IN HUMAN SERA USING HSV TYPE 1 AND TYPE 2 POLYSPECIFIC ANTIGENS BLOCKED WITH TYPE HETEROLOGOUS RABBIT ANTIBODIES

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Department of Clinical Virology Institute of Medical Microbiology University of Copenhagen Denmark

Vestergaard B F & Grauballe P C ELISA for herpes simplex virus (HSV) type specific antibodies in human sera using HSV type 1 and type 2 polyspecific antigens blocked with type heterologous rabbit antibodies Acta path microbiol scand Sect B 87 261 263 1979

Crude antigenic preparations made from rabbit cornea cells infected with either HSV type 1 or type 2 could be used in ELISA for titration of HSV type specific antibodies in human sera After immunochemical binding of the crude HSV antigens in microtitre wells by use of type homologous rabbit antibodies type common antigenic sites were blocked with type heterologous rabbit antibodies Titration of human sera in this system showed that high concentrations of type heterologous rabbit antibodies were capable of completely blocking type-common antigenic sites while leaving type specific antigenic sites unblocked and capable of reacting with human antibodies Thus HSV type specific antibodies in human sera could be measured in ELISA without the use of purified typespecific antigens

Key words Herpes simplex virus ELISA type specific antibodies

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Received 16 ii 79 Accepted 15 v 79

A number of existing serological methods based on relative measurement of antibody activity against herpes simplex virus (HSV) type 1 and type 2 are in general unable to exactly identify and measure antibodies against both HSV types in the same serum (3)

We have previously developed an ELISA for titration of HSV antibodies (4) and have reported on the measurement of HSV type 2 specific antibodies by ELISA using type 2 specific antigens purified by immunospecific affinity chromatography (1)

This paper describes an ELISA method for exact measurement of both HSV type 1 and type 2 specific antibodies in human sera without the use of purified HSV type specific antigens

Materials and Methods

Antigens Crude HSV type 1 (strain F) and HSV type 2 (strain G) antigenic preparations were prepared from

non ionic detergent Triton X 100 as previously described (5)

Antibodies Rabbits were injected intracutaneously with the total sonicate from 10^7HSV infected rabbit cornea cells maintained in medium supplemented with 2% rabbit serum and harvested at the time of full cytopathic effect The animals were boosted twice at intervals of two weeks and bled 10 days after the last booster Each serum was evaluated by quantitative immunelectrophoresis

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purified polyspecific rabbit immunoglobulins to HSV type 1 and HSV type 2 will be designated Ra 1 and Ra 2 respectively

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was fixed to the plastic with glutaraldehyde as described previously for antigen coating (4) 3) *Protein saturation* 200 µl of 10% foetal calf serum diluted in the washing fluid was added to each well and left overnight at 4°C All the subsequent immunochemical reactions (steps 4 to 8) took place in PBS with 0 05% Tween 20 0 5% bovine albumin and additional NaCl up to 0 5 M 4) *Antigen coating* 100 µl crude HSV type 1 antigen dilutions were added to Ra 1 coated wells and HSV type 2 antigen to Ra 2 coated wells The antigens were left overnight at 4°C 5) *Blocking* 100 µl of Ra 2 dilutions

sera 100 µl of human serum diluted 1 100 was added to each well without emptying the wells for blocking rabbit antibodies Incubation for 30 minutes at 37°C 7) *Enzyme* 100 µl of peroxidase conjugated rabbit anti human IgG (p 1090 DAKO immunoglobulins Copenhagen Denmark) diluted 1 1000 was added to each well and incubated for 30 minutes at 37°C 8) *Stain* 50 mg of 1 2 phenylendiaminodihydrochloride (Fluka AG pu riss) was dissolved in 100 ml 0 05 M Na_2HPO_4 –0 0?4 M citric acid buffer pH 5 0 Just before use 20 µl of 30% H_2O_2 was added and 100 µl of the mixture was placed in each well The reaction was stopped after 5 to 15 minutes by the addition of 150 µl 2 N H_2SO_4 and the optical density (OD) values were read at 49? nm in a Vitatron photometer Extensive washings were carried out between steps 2 to 5 and 6 to 8 using PBS with 0 05% Tween 20 and additional NaCl up to 0 5 M

Results and Discussion

Fig 1 shows the almost linear decrease in OD values with decreasing amounts of antigen Fig 1A shows that Hu 1 reacted more strongly than Hu 2 with HSV type 1 antigen while the opposite pattern is seen with HSV type 2 antigen (Fig 1B) The arrows indicate the amount of antigen used in the HSV type specific blocking assay shown in Fig 2

Fig 2A shows that Ra 2 in dilutions 1 8 and 1 16 was capable of blocking completely the HSV type-common antigenic sites of the crude HSV type 1 antigen since Hu 2 did not give any specific reactions In the same dilution range of blocking antibodies Hu 1 reacted positively thus indicating that HSV type 1 specific antigenic sites were accessible to human antibodies Fig 2B shows that similar results were obtained with HSV type 2 antigens blocked with Ra 1 The reason why the actual OD values of Hu 1 and Hu 2 without blocking antibodies were higher in Fig 2 than those indicated by the arrows in Fig 1 was that longer reaction times were used in the blocking assay This did not lead to an increase in the OD level obtained with Hu 0

In conclusion Fig 2 shows that blocking with high concentrations of Ra 1 and Ra 2 resulted in a completely HSV type specific ELISA for titration of human antibodies A number of individual human sera obtained from virologically investigated cases or sera titrated by other serological methods have been tested in our ELISA using adjusted amounts of crude HSV type 1 and 2 antigens and of blocking Ra 1 and Ra 2 determined as described above The results so far have been in agreement with the clinical records or with previous serological results

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This work was supported by the *Danish Cancer Society* and *Konsul Ehrenfried Owesen & Hustrus Fond* We thank Dr *I Ørstavik* for supplying the HSV type 2 antiserum from the neonatal herpes patient and Ms *Hanne Spanggaard* and Ms *Ulla Søborg Larsen* for excellent technical assistance

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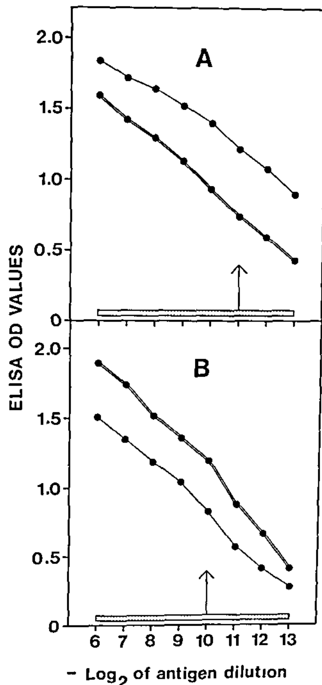


Fig 1 ELISA OD values of Hu 1 Hu 2 and Hu 0 diluted 1 100 reacting with two fold dilutions of HSV type 1 antigen (Fig 1A) and HSV type 2 antigen (Fig 1B) without blocking rabbit antibodies
Hu 1 ———— Hu 2 ════

The shadded area represents the range of OD values (0 04–0 08) obtained with Hu 0

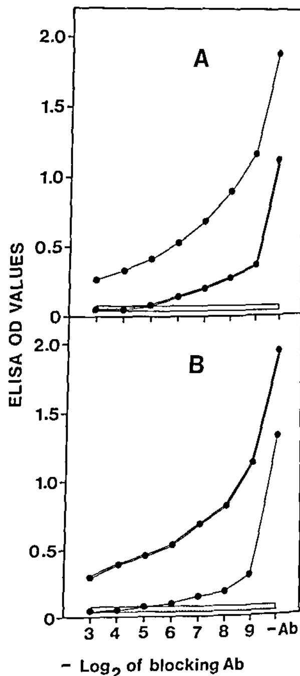


Fig 2 ELISA of Hu 1 Hu 2 and Hu 0 diluted 1 100 reacting with HSV type 1 antigen diluted 1 2048 and blocked with two fold dilutions of Ra 2 (Fig 2A) and HSV type 2 antigen diluted 1 1024 and blocked with two fold dilutions of Ra 1 (Fig 2B)
Hu 1 ———— Hu 2 ════

The shaded area represents the range of OD values (0 04–0 08) obtained with Hu 0

from a one year old child which had survived neonatal herpes and several minor recurrencies and two reconvalescent sera from primary infections in adults All three cases were virologically confirmed HSV type 2 infections and none of the sera reacted in ELISA with a purified HSV type 1 specific glycoprotein (*Vestergaard & Grauballe* in preparation)

A third serum pool (Hu 0) was a collection of sera without detectable HSV antibodies determined by ELISA (4)

ELISA The test was made in microtest plates (96 U PS NUNC Roskilde Denmark) 1) *Pre coating* was performed as described previously (4) 2) *Catching antibodies* Ra 1 or Ra 2 diluted 1 500 in distilled water

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QUANTITATIVE MICROBIOLOGICAL ASSAY OF THIOMERSAL USING AGAR DIFFUSION FROM PAPER DISCS

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Statens Seruminstitut, Serum and Vaccine Department and Biostatistical Department Copenhagen Denmark

Christensen T E & Bentzon M W Quantitative microbiological assay of thiomersal using agar diffusion from paper discs Acta path microbiol scand Sect B 87 265 269 1979

A quantitative assay method for determination of thiomersal in biological products is described and evaluated A microbiological method using agar diffusion from filter paper discs is used The advantages of the method in terms of low cost and reliability for determining the actual antimicrobial activity in the preparation are discussed The assay method is in routine use in the production control of vaccines at our Institute

Key words Thiomersal quantitative assay agar diffusion paper discs

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Various methods for the evaluation of the antimicrobial activity of preservatives are in use in several laboratories Detailed descriptions of such microbiological assays have been presented by *Greenberg & Naubert* 1970 who also stress the advantages of microbiological assays over chemical assays as regards evaluation of the antimicrobial activity of preservatives in pharmacological products

In the production control at Statens Seruminstitut Copenhagen the content of thiomersal in biological products has been determined for many years by chemical methods However being aware of the shortcomings of the chemical assays in estimation of the actual antimicrobial activity of preservatives we considered several years ago adapting the same technique as that used in the paper disc agar diffusion assay of antibiotic activity described by *Vesterdal* 1947 *Thomsen* 1967 and *Bang* 1970

Following preliminary attempts which gave promising results the method described below was designed

MATERIALS AND METHODS

The preparations to be assayed consisted of formaldehyde inactivated antigen aluminium hydroxide and thiomersal stabilized with EDTA*)

After suitable dilution the test preparation was sucked up into filter paper discs which where placed together with discs containing reference solutions on a petri plate containing broth agar The plates were left for prediffusion at room temperature for 24 hours The discs were then removed and the plates flooded with 5 ml of a suspension of the test bacteria *After sucking off* the surplus the plates were incubated at 37 °C for 24 hours and the inhibition zones were measured using a strip of millimeter paper

The nutrient medium used was common broth agar

The petri plates used were of plastic material and were flat bottomed with a diameter of 14 cm

The broth agar plates were manufactured on a large scale and at low cost, and therefore the amount of agar in each plate unfortunately varies The size of the *inhibition* zone depends on the thickness of the agar layer so discs with each of the reference solutions must be placed on

*) Disodium salt of ethylenediamine tetraacetic acid

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each plate The plates must be free from bubbles and not too wet when used

The filter paper discs are obtained commercially (Schleicher & Schull No 2668) and are autoclaved before use The weight of water adsorbed by each of 100 discs from each batch was measured From the batch used in this investigation the mean weight absorbed was 31 4 mg with standard deviation 2 0 mg

The bacterial suspension used for inoculum was a 24 hour broth culture of Sarcina lutea ATCC No 9341 diluted 1 3 in saline

Preliminary Assays

Four discs representing a two fold dilution series of thiomersal in saline from 1 40 000 to 1 320 000 were placed on each of five plates The plates were left for prediffusion at room temperature for 24 hours after which the discs were removed and the plates inoculated and incubated as mentioned under Materials and Methods

In addition five other plates were inoculated as described Five discs representing a two fold dilution series of thiomersal in saline from 1 10 000 to 1 160 000 were placed on each plate and the plates were incubated immediately for 24 hours (i e without a prediffusion period)

Following the incubation period all inhibition zones were read

Dilutions of thiomersal in saline concentrations 1 80 000 1 160 000 1 320 000 and 1 640 000 were prepared Equal solutions were produced with EDTA EDTA and aluminium hydroxide and EDTA aluminium hydroxide and horse serum were added in concentrations which were one tenth of those present in the vaccines to be tested Horse serum was chosen in order to see how the presence of protein affected the log concentration response curve These 16 preparations were compared on 12 plates with four preparations on each using prediffusion for 24 hours

Proposed Procedure

On the basis of these assays the following procedure for determining the concentration of thiomersal in vaccines was developed

The concentration of the standard solutions and the strain of test bacteria were selected so as to be optimal in a concentration range of thiomersal 50 to 100 mg/litre since the vaccines are intended to contain 100 mg/litre (1 10 000)

Two standard solutions of thiomersal 1 80 000 (12 5 mg/litre) and 1 160 000 (6 25 mg/litre) in saline are used

The preparations to be tested are diluted 1 10 in saline

Test and standard solutions are sucked up into the filter paper discs and one disc with each of the standard solutions is placed on each plate together with two discs containing test solution

After this the plates are left at room temperature for 24 hours for prediffusion

The discs are removed and the plates flooded with 5 ml of a suspension of the test bacteria This is allowed to settle for one minute and the surplus is sucked off

The plates are incubated for 24 hours at 37 °C and the zones of inhibition are measured

For each plate the zone diameter corresponding to the standard solutions is plotted against the log of concentration of thiomersal From this standard curve the log of concentration of thiomersal in the test solution is calculated as shown in Fig 1

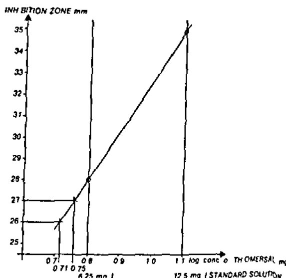


Fig 1 Example of calculation of a plate from an assay of *tetanus vaccine (Table 2)*
Inhibition zones Standard 12 5 mg/l – 35 mm 6 25 mg/l – 28 mm
Test Vaccine diluted 1 10 27 mm corresponding to 0 75 log units and 26 mm corresponding to 0 71 log units Mean 0 73 indicating that the undiluted vaccine *contained 54 mg/l*

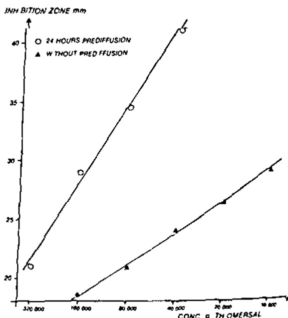


Fig 2 Relationship between a two fold dilution series of thiomersal in saline and inhibition zones with and without 24 hour prediffusion

TABLE 1 *Results of an Assay to Determine the Effect of EDTA Aluminum Hydroxide and a Small Amount of Protein on the Activity of Thiomersal*

Plate No	Preparation	Zone diameter	Preparation	Zone diameter	Preparation	Zone diameter	Preparation	Zone diameter
1	M1	39	M2	34	M3	26	M4	18
2	M1	41	M2	36	M3	29	M4	17
3	T1	38	T2	32	T3	24	T4	17½
4	T1	41	T2	35	T3	28	T4	19
5	A1	41	A2	33	A3	28	A4	20
6	Failure							
7	P1	42	P2	36	P3	26	P4	16
8	P1	39½	P2	33	P3	25	P4	15
9	M1	41	T2	34	A3	27	P4	17
10	M2	32½	T3	24	A4	13	P1	39
11	M3	28	T4	21	A1	42	P2	35
12	M4	20	T1	42	A2	36	P3	26

M Thiomersal
T Thiomersal + EDTA
A Thiomersal + EDTA + Aluminium hydroxide
P Thiomersal + EDTA + Aluminium hydroxide + 1% Horse protein
M1 T1 A1 P1 Thiomersal 1 80 000
M2 T2 A2 P2 Thiomersal 1 160 000
M3 T3 A3 P3 Thiomersal 1 320 000
M4 T4 A4 P4 Thiomersal 1 640 000

RESULTS

Preliminary Assays

The relationship between the concentration of a constituents of the type of test preparation to a two fold dilution series of thiomersal For the first eight plates in the assay, the variance due to differences between plates could not be separated from that due to differences between preparations Therefore the last four plates were designed as a Latin square The conclusions based on both experiments are as follows

1) The inhibition zones for preparations with the

TABLE 2 *Results of Assay of One Vial of Tetanus Vaccine on Ten Agar Plates*

Plate No	Standard 1 80 000	Standard 1 160 000	Tetanus vaccine 1 10	Log conc	Tetanus vaccine 1 10	Log conc
1	36	30	28	1 71	26	1 60
2	35	28	27	1 75	26	1 71
3	34	28	26	1 70	27	1 75
4	38 5	31	30 5	1 77	29	1 71
5	39	31 5	30 5	1 78	30	1 73
6	37	28	27 5	1 78	28	1 80
7	32	25	22	1 63	26	1 84
8	37	30	28	1 73	28	1 73
9	35 5	29 5	27 5	1 70	27 5	1 70
10	35	28	27 5	1 78	27 5	1 78

For figures see text
Mean value 1 74 corresponding to 55 mg/litre
Standard deviation 0 053 corresponding to 13%

same concentration of thiomersal were not influenced by adding EDTA aluminium hydroxide and protein

2) The variance between plates is significant

3) The standard deviation of the zone diameters is estimated at 1 26 mm

4) The log concentration inhibition zone curve calculated from the results of Table 1 is slightly curved the slope being about 7 6 mm per dilution step for the middle part and about 9 1 mm and 6 1 mm for the lower and upper parts of the curve respectively

Proposed Procedure

A vial of tetanus vaccine was investigated on ten plates by the method described

Table 2 shows the inhibition zones given by the standard solutions and the test solutions and the calculated value of the log to the concentration of thiomersal in the vaccine in mg/litre The mean value observed is 1 74 corresponding to 55 mg/litre and the standard deviation 0 053 corresponding to 13%

Using the estimate of the slope (7 6/0 30) obtained from the results of the preliminary assay the standard deviation of the log of the concentration (X) can be calculated as

$$SD(X) = 0\ 30 \times \frac{1\ 26}{7\ 6} \times \sqrt{1 + \frac{1}{2} + \frac{(X - 1\ 95)^2}{0\ 045}}$$

By substituting for X the mean value 1 74 from Table 2 SD(X) becomes 0 078 or somewhat higher than the observed standard deviation The standard deviation obtained by the above formula attains its minimum value SD(X) = 0 061 when X = 1 95

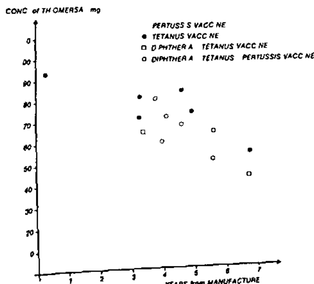


Fig 3 Activity of thiomersal in relation to storage of a few old vaccines kept at 4 °C for up to 7 years

i e when the observed zone for the test preparation equals the average of the zones obtained for the standard preparations

The assay method was tried on a few old vaccines which had been stored in the Department at 4 °C for up to 7 years

Fig 3 shows the results which indicate that the activity of thiomersal disappears with a half life of 7 years

DISCUSSION

As compared to chemical assays for thiomersal the method described consumes less time and ressources when a large number of uniform samples are to be analysed and compared

Though chemical tests for thiomersal are usually more accurate microbiological assays measure more reliably the antimicrobial activity of thiomersal in the sample since there is the possibility that thiomersal is inactivated without loss of the determinant measured in the chemical assay

The use of mass produced agar plates makes the assay easy to set up and cheap to perform but the accuracy and sensitivity are reduced The use of specially prepared antibiotic assay medium would give better results but at this Institute it is found that the specificity and accuracy of the assay as described in this paper are fully sufficient

In selecting the test bacteria emphasis was placed on the importance of the inhibition zones being clearly defined Other far more sensitive strains exist but the use of any of these was not considered an advantage since the sensitivity of the selected strain is sufficient for our purpose

Using prediffusion the log concentration inhibition zone curve has a higher slope viz about 7 mm per doubling of concentration against 3 mm without prediffusion This gives the test a higher power of resolution but also increases the deviation of the points from the standard curve The balance between this increase and the increase in slope determines the overall sensitivity of the assay However the crucial point in using prediffusion is that the larger zones are technically easier to measure

The assay method is now in routine use in the production control of vaccines

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Acta path microbiol scand Sect B 87 271–276 1979

RAPID IDENTIFICATION OF *ENTEROBACTERIACEAE*

II Use of a β glucuronidase Detecting Agar Medium (PGUA Agar) for the Identification of E coli *in Primary Cultures of Urine Samples*

M KILIAN and P BÜLOW

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Kilian M & Bulow P Rapid identification of *Enterobacteriaceae* 2 Use of a β glucuronidase detecting agar medium (PGUA agar) for the identification of *E coli* in primary cultures of urine samples Acta path microbiol scand Sect B *87* 271–276 1979

β glucuronidase activity is an exclusive characteristic of *E coli* and some shigellae among *Enterobacteriaceae* and *Vibrionaceae* An agar medium (PGUA agar) which permits the detection of bacteria with β glucuronidase activity in mixed cultures was evaluated as a primary culture medium for clinical samples of urine The medium was selective for enterobacteria and yielded significantly higher recoveries than MacConkey agar Based on the examination of 3 460 urine samples it was found that the use of the PGUA agar has several advantages over conventional methods 1) 94% of all *E coli* cultures could be identified on the basis of their appearance on the primary plates 2) The use of the PGUA medium resulted in a 46% reduction in the cost of media employed and a 67% reduction in the time required for the processing of urine samples

Key words *Enterobacteriaceae Escherichia coli* urinary tract infection rapid identification β-glucuronidase

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The diagnostic work in a clinical bacteriological laboratory is a time-consuming and costly process A simplification of this process is desirable not only

concerned with the identification of enterobacteria Thus the majority of microorganisms implicated in urinary tract infections belong to this group of bacteria and consist principally of one single species viz *Escherichia coli* In fact it has been reported that over 80% of uncomplicated cases of urinary tract infections are caused by bacteria belonging to

Because of much overlapping of characteristics the identification of enterobacteria requires the performance of a considerable number of biochemical tests However in a recent study of glycosidase activities among enterobacteria (1) it was demonstrated that β glucuronidase activity is an exclusive characteristic of *E coli* and some *Shigella* species among all the species of *Enterobacteriaceae* and *Vibrionaceae* The high frequency of this characteristic observed among *E coli* strains (97%) indicated that the β-glucuronidase test (PGUA test) has a valuable potential for simplifying the differentiation of *E coli* from other enterobacteria

The present paper describes an agar medium (PGUA agar) which permits the detection of

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The diagnostic work in a clinical bacteriological laboratory is a time-consuming and costly process A simplification of this process is desirable not only in order to reduce costs but also to allow for the fastest possible initiation of patient therapy

A considerable part of the diagnostic work is concerned with the identification of enterobacteria Thus the majority of microorganisms implicated in urinary tract infections belong to this group of bacteria and consist principally of one single species

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The present paper describes an agar medium (PGUA agar) which permits the detection of

TABLE 1 *Components of PGUA Agar Medium*

Per litre water	
Proteose peptone nr 3 (Difco)	10 g
Yeast extract (Difco)	5 g
Kaolin powder (washed)	20 g
Japan agar powder (Nordisk Droge)	11 g
KH_2PO_4	33 44 mg
Na_2HPO_4 2 H_2O	11 83 g
4 nitrophenyl β D glucopyrano siduronic acid (Merck)	0 4 g
Maranil A 75 (Henkel & Co) 3 3 ml of a 5% aqueous solution	
pH 8 0	

bacteria with β glucuronidase activity in mixed cultures This medium is considered suitable as a primary medium for the cultivation and examina tion of clinical samples of urine in a routine bacteriological laboratory

MATERIALS AND METHODS

Composition of PGUA agar

The constituents of the PGUA agar are listed in Table 1 4 nitrophenyl β D glucopyranosiduronic acid (Merck) is the chromogenic substrate for β glucuronidase The concentration of the compound (0 4 g/l) was the minimum amount that resulted in definite yellow colonies of bacteria with β glucuronidase activity Kaolin powder provided the medium with an opaque white background for stained colonies The sodium salt of dodecylsulphate sulphonate (Maranil A 75 Henkel and Co Dusseldorf) was added to inhibit swarming of *Proteus* species

Evaluation of the PGUA agar

The ability of the PGUA agar to support growth of selected microorganisms was compared with that of MacConkey agar and 5% blood agar The evaluation included two isolates of each of the species *Escherichia coli Klebsiella pneumoniae Staphylococcus aureus S epidermidis Streptococcus faecalis* and *Candida albicans* Washed saline suspensions of cells harvested from overnight broth cultures were diluted in 10 fold series with sterile saline Appropriate dilutions were plated on triplicate sets of each of the three media The recoveries were recorded after incubation for 24 hours at 35°C

In order to evaluate the PGUA agar as a medium for the identification of *E coli* it was used as primary medium for the cultivation of urine samples in the Department of Clinical Bacteriology Statens Seruminsti tut Municipal and University Hospital of Århus During a 4 month period 9 247 clinical samples of urine submitted to the laboratory for microbiological examina tion were processed by the conventional procedure of the laboratory and by an alternative procedure which included the PGUA medium Each of the procedures was carried out independently by different laboratory techni cians Only 3 460 (37 4%) of these samples which on primary media yielded growth corresponding to more than 100 000 microorganisms per ml of urine (signifi cant bacteriuria) were included for further study

The two procedures are illustrated schematically in Fig 1 The conventional procedure comprised initial cultivation of samples on 5% blood agar (quantitative plating) and on two plates of MacConkey agar used for quantification and spreading respectively After over night incubation representative colonies were subculti vated for purification if required Pure cultures of enterobacteria were transferred to a set of biochemical test media lactose agar nutrient agar and semisolid broth indole Voges Proskauer malonate glucose ornithine lysine arginine gelatine nitrate and ONPG media After overnight incubation the isolates were

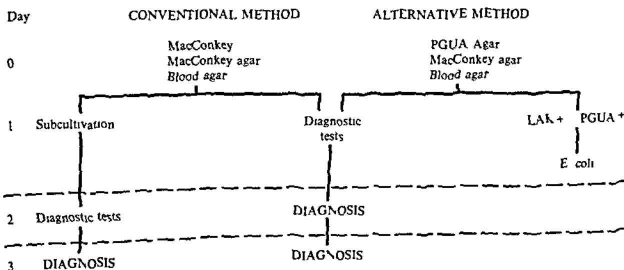


Fig 1 Conventional and alternative (PGUA) procedures for the microbiological examination of clin cal samples of urine

identified on the basis of the results of these tests Further biochemical tests were performed if required

Using the alternative procedure all urine samples were cultivated on 5% blood agar MacConkey agar (quantification) and PGUA agar (spreading) After incubation for 20–24 hours bacteria showing the combination of yellow colonies on PGUA agar (β glucuronidase producers) and red colonies on MacConkey agar (lactose fermenters) were identified as *Escherichia coli* According to the complete alternative procedure (Fig 1) all remaining isolates would be examined further using conventional tests However since this would not add to the evaluation of the PGUA medium this last step was not carried out.

The bacterial diagnoses obtained by the two procedures were compared at weekly intervals Isolates identified differently by the two procedures were stored as agar stabs for reexamination by an extended series of biochemical tests and serology

RESULTS

Growth

The ability of the PGUA medium to support growth of selected strains of enterobacteria staphylococci streptococci and candida is illustrated in Table 2 The table shows the recoveries on PGUA agar and MacConkey agar expressed as percentage of the recovery on 5% blood agar plates PGUA agar appeared to be more selective for enterobacteria than MacConkey agar as it did not support growth of strains of staphylococci enterococci and candida However the recovery of strains of enterobacteria on PGUA agar was significantly higher than on MacConkey agar

PGUA Reaction

The incubation time for PGUA plates permitting optimal differentiation between bacteria with and without β glucuronidase activity was 18–24 hours Shorter incubation resulted in reduced colour intensity of colonies With prolonged incubation a widening of the yellow zones around colonies of β glucuronidase producing bacteria was observed thus causing difficulty in relating the activity to individual colonies

Isolates from urine samples

During examination of the 3 460 urine samples by the conventional procedure 3 476 bacterial strains were isolated for identification as shown in Table 3 The figures in the table represent the final data which are corrected after reexamination of selected strains (see below) The majority of isolates (2 824 = 81%) belonged to the family *Enterobacteriaceae* *E coli* being the single species isolated most frequently (2 004 isolates = 71% of all *Enterobacteriaceae* strains) *E coli* was present in pure culture in 1 657 samples corresponding to 48% of all samples yielding significant growth

Comparative Evaluation of the Two Identification Procedures

By the alternative (PGUA) procedure 1 892 cultures were identified directly as *E coli* on the basis of the reactions observed on the primary plates (positive PGUA and lactose fermentation reactions) Comparison with the results obtained by the vonventional procedure revealed that 15 of these 1 892 cultures had not been identified as *E coli* by the standard biochemical tests However reexamination of these strains by an extended number of biochemical tests and serology confirmed their identity as *E coli* Furthermore 118 cultures identified as *E coli* by standard biochemical tests remained unidentified by the PGUA method since they were all PGUA negative Reexamination of

TABLE 2 *Comparative Recoveries from Suspensions of Selected Microorganisms on Blood Agar PGAU-agar and MacConkey Agar*

	Recovery in per cent of that on blood agar		
	Blood agar	PGUA agar	MacConkey agar
Escherichia coli strain I	100%	80 3%	69 6%
E coli strain II	100%	88 5%	52 2%
Klebsiella pneumoniae strain I	100%	106 2%	87 6%
K pneumoniae strain II	100%	111 6%	93 7%
Staphylococcus epidermidis	100%	<0 1%	87 2%
S aureus	100%	<0 1%	<0 1%
Streptococcus faecalis strain I	100%	<0 1%	91 0%
S faecalis strain II	100%	<0 1%	95 9%
Candida albicans strain I	100%	<0 1%	98 9%
C albicans strain II	100%	<0 1%	82 6%

TABLE 1 *Components of PGUA Agar Medium*

Per litre water	
Proteose peptone nr 3 (Difco)	10 g
Yeast extract (Difco)	5 g
Kaolin powder (washed)	20 g
Japan agar powder (Nordisk Droge)	11 g
KH_2PO_4	33 44 mg
Na_2HPO_4 2 H_2O	11 83 g
4-nitrophenyl-β-D-glucopyranosiduronic acid (Merck)	0 4 g
Maranil A 75 (Henkel & Co), 3 3 ml of a 5% aqueous solution	
pH 8 0	

bacteria with β-glucuronidase activity in mixed cultures This medium is considered suitable as a primary medium for the cultivation and examination of clinical samples of urine in a routine bacteriological laboratory

MATERIALS AND METHODS

Composition of PGUA-agar

The constituents of the PGUA-agar are listed in Table I 4-nitrophenyl-β-D glucopyranosiduronic acid (Merck) is the chromogenic substrate for β-glucuronidase The concentration of the compound (0 4 g/l) was the minimum amount that resulted in definite yellow colonies of bacteria with β-glucuronidase activity Kaolin powder provided the medium with an opaque, white background for stained colonies The sodium salt of dodecylsulphate sulphonate (Maranil A 75 Henkel and Co , Dusseldorf) was added to inhibit swarming of *Proteus* species

Evaluation of the PGUA-agar

The ability of the PGUA-agar to support growth of selected microorganisms was compared with that of MacConkey agar and 5% blood agar The evaluation included two isolates of each of the species *Escherichia coli, Klebsiella pneumoniae Staphylococcus aureus S epidermidis, Streptococcus faecalis* and *Candida albicans* Washed saline suspensions of cells harvested from overnight broth cultures were diluted in 10-fold series with sterile saline Appropriate dilutions were plated on triplicate sets of each of the three media The recoveries were recorded after incubation for 24 hours at 35°C

In order to evaluate the PGUA-agar as a medium for the identification of *E coli*, it was used as primary medium for the cultivation of urine samples in the Department of Clinical Bacteriology, Statens Seruminstitut, Municipal and University Hospital of Århus During a 4-month period, 9,247 clinical samples of urine submitted to the laboratory for microbiological examination were processed by the conventional procedure of the laboratory and by an alternative procedure which included the PGUA medium Each of the procedures was carried out independently by different laboratory technicians Only 3,460 (37 4%) of these samples, which on primary media yielded growth corresponding to more than 100,000 microorganisms per ml of urine (»significant bacteriuria«), were included for further study

The two procedures are illustrated schematically in Fig 1 The conventional procedure comprised initial cultivation of samples on 5% blood agar (quantitative plating), and on two plates of MacConkey agar used for quantification and spreading, respectively After overnight incubation, representative colonies were subcultivated for purification, if required Pure cultures of enterobacteria were transferred to a set of biochemical test media lactose agar, nutrient agar and semisolid broth, indole, Voges-Proskauer, malonate, glucose ornithine, lysine, arginine gelatine, nitrate and ONPG media After overnight incubation, the isolates were

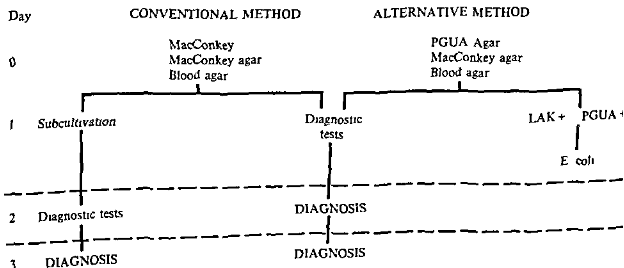


Fig 1 Conventional and alternative (PGUA) procedures for the microbiological examination of clinical samples of urine

TABLE 5 *β glucuronidase Activity of 123 Shigella strains*

	No of strains	β glucuro nidase producers
S dysenteriae	13	7 (54%)
S boydi	6	1 (17%)
S flexneri	41	12 (29%)
S sonnei	63	62 (98%)
Total	123	82 (67%)

faecal isolates kindly provided by Dr *K Gaarslev* (Statens Seruminstitut Copenhagen) Of these 82 strains (67%) produced yellow colonies on PGUA agar Detailed results are shown in Table 5

Serology of PGUA negative E coli *Strains*

All 94 *E coli* strains which lacked β glucuronidase activity were serotyped using the designated typing sera of the International Escherichia Reference Centre (WHO) Twenty five of these strains (27%) had the antigenic composition 01 Kl H An additional 12 strains that were rough and non flagellated also possessed a serotype Kl capsule None of these 37 Kl strains fermented adonitol dulcitol or sucrose but varied in their capacity to ferment xylose and sorbose The majority of the strains were negative or weakly positive in the indole test

Ten of the remaining strains which were non flagellated reacted with 075 antiserum and possessed a polysaccharide K antigen which however did not react in any of the designated typing sera Another 10 strains were rough and non flagellated The remaining 30 strains were distributed on 25 different serotypic combinations

TABLE 6 [illegible]

Conventional procedure	
2 824 isolates @ 6 35 min	17 932 min
Alternative procedure	
PGUA negative isolates	
93? isolates @ 6 35 min	5 918 min
Relative saving in time by the use of alternative procedure	12 014 min (67%)

Comparison of Time Required and Cost of Media using the Two Procedures

Time analysis of the individual steps included in the conventional processing of several hundred strains showed that the time required for complete examination of one enterobacterium isolate was 6 35 min Based on all the urine samples included in the study the time saved by the use of the alternative procedure was 67% as compared to the conventional procedure (Table 6)

The production cost of media required for the conventional identification of enterobacteria (see Materials and Methods) was calculated to be DKr 14 02 per strain The extra cost of para nitrophenyl β D glucupyranosiduronic acid per PGUA agar plate used in the alternative procedure was DKr 0 77 On the basis of these figures the total percentage saving in cultivation and identification media was 46% using the alternative procedure The calculation was carried out on the basis of all 3 460 samples which yielded significant growth

DISCUSSION

Our previous finding that β glucuronidase activity is an exclusive character of *Escherichia coli* and some *Shigella* species among *Enterobacteriaceae* and *Vibrionaceae* (1) is confirmed by this study Out of 2 004 urine isolates of *E coli* 94% showed β glucuronidase activity In contrast none of 1 295 strains representing the genera *Aeromonas Citrobacter Edwardsiella Enterobacter Erwinia Hafnia Klebsiella Proteus Salmonella Serratia Vibrio* and *Yersinia* examined in this and our previous study (1) possessed this enzyme activity These results point to the β-glucuronidase activity as a highly distinguishing characteristic

The PGUA agar described in this paper permits the detection of β-glucuronidase-producing bacteria in mixed cultures Although this plate method appeared to be somewhat less sensitive than the suspension method described previously (1) 94 4% of the 2 004 *E coli* [illegible]
as β gluc[illegible]
the agar [illegible]
selective [illegible] MacConkey agar
In addition the recovery of enterobacteria was higher than on MacConkey agar and was comparable to that on blood agar

The primary aim of the study was to evaluate this PGUA agar as a means of identifying *E coli* in clinical samples of urine without the necessity for using further identification tests The evaluation was based on the simultaneous processing of 3 460 urine samples with significant growth by our conventional method and by an alternative proce

TABLE 3 *Identity of 3,476 Isolates from 3,460 Samples of Urine Yielding Significant Growth*

Genera/species	No of isolates
Enterobacteriaceae	
Escherichia coli	2 004
Klebsiella pneumoniae	286
K oxytoca	81
K ozaenae	1
K rhinoschleromatis	1
Enterobacter cloacae	58
E aerogenes	10
Citrobacter freundii	23
C koseri	16
Proteus mirabilis	230
P vulgaris	75
P morgani	23
P rettgeri	2
P inconstans	9
Unidentified enterobacteria	5
	2 824
Other taxa	
Streptococcus faecalis/faecium	370
Streptococcus pyogenes	12
Non-haemolytic streptococci	11
Staphylococcus aureus/epidermidis	110
Pseudomonas aeroginosa	112
Acinetobacter	21
Candida	16
	652
Total	3 476

these strains by serology and an extended number of biochemical tests showed that 112 were PGUA negative strains of *E coli* The remaining six strains could not be confirmed as *E coli* One of the isolates was a *Citrobacter* strain, and the remaining five strains were atypical strains which could not be assigned definitively to any of the known species

To summarize, comparison of the two methods (Table 4) revealed that all 1,892 cultures identified as *E coli* by the PGUA method on the basis of their appearance on the primary plates were correctly identified Another 112 cultures of *E coli* (5 6%) remained unidentified after initial examination of the plates because of being PGUA negative The number of strains misidentified by the conventional procedure was 1 0% (21 strains) of the total number of strains for which identification data were available by both methods

Frequency of PGUA Reaction among E coli *and* Shigella *Strains*

As mentioned above, 112 (5 6%) out of a total of 2,004 cultures of *E coli* failed to produce yellow colonies on the PGUA agar medium Reexamination of the β glucuronidase activity of these strains by use of the suspension method described previously (1) *showed enzyme activity in 18 of these 112* strains

In order to determine the frequency of β glucuronidase activity among shigellae, 123 strains representing four species were examined on PGUA agar medium (Table 5) These included 14 strains from the National Collection of Type Cultures (Colindale, London) and 109 laboratory cultures of

TABLE 4 *Summary of the Result of* Escherichia coli *Diagnosis by a Conventional Method and the PGUA method (=Alternative Method)*

Identification procedure	Strains identified as *E coli* following primary examination	Comments on primary examination	Strains identified as *E coli* following reexamination	Final comments
Conventional	1 995	− 6 non *E-coli* (all PGUA −) + 15 *E coli* misidentified as non *E coli* (all PGUA +)	2 004	1% of strains misidentified on primary examination
Alternative (PGUA method)	1,892	All confirmed	1,892	94% of *E coli* rapidly and correctly identified

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AN ULTRASTRUCTURAL STUDY ON THE EXCYSTATION OF THE SPOROZOITES OF *TOXOPLASMA GONDII*

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Ferguson D J P Birch Andersen A Siim J Chr & Hutchison W M An ultrastructural study on the excystation of the sporozoites of *Toxoplasma gondii* Acta path microbiol scand Sect B 87 277-283 1979

The ultrastructural changes which occur during the *in vitro* excystation of the sporozoites of *Toxoplasma gondii* were examined The excystation was carried out at 37° C on suspensions of oocysts which had been ground and then treated with an excysting medium containing 0 25% trypsin and 0 75% sodium taurocholate in phosphate buffered saline pH 7 3 It was found that sporocysts within intact oocysts were unaffected while sporocysts exposed to the medium ruptured The sporocyst wall consisted of two layers and during excystation the four plates which form the inner layer started to curl inward At the same time changes were seen at the specialized junctions between these plates When the junctions finally break the plates separate The outer layer of the sporocyst wall is then ruptured at points directly above where the plates were joined Each of the four portions of the sporocyst wall curled inward to form a tightly wound whorl The sporozoites can escape through the openings created between the portions of the sporocyst wall

Key words *Toxoplasma gondii* excystation sporozoite ultrastructure

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Received 9 i 79 Accepted 9 iv 79

The coccidian parasite *Toxoplasma gondii* undergoes sexual development in the small intestine of the cat When the oocysts which are produced sporulate they are morphologically similar to those of the genus *Isospora* Ultrastructural observations on the *in vitro* excystation of sporozoites of *Isospora* spp has been limited to *I felis* (12) and *I endocallimici* (13) In this paper the ultrastructural changes which occur during the excystation of the sporozoites of *T gondii* will be described and compared to those reported for other members of the coccidia

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MATERIALS AND METHODS

Oocysts were concentrated from the faeces of specific pathogen free cats which had been infected by feeding

this study were approximately 2 months old After removal of the potassium dichromate the oocysts were treated with 6% sodium hypochlorite for 15 minutes washed in water and then *suspended* in phosphate buffered saline (PBS) pH 7 3 At this stage the oocysts were ground to rupture the oocyst wall The excysting medium contained trypsin and sodium taurocholate dissolved in PBS in such amounts that the *final* concentration in the oocyst suspension was 0 25% (w/v) trypsin and 0 75% (w/v) sodium taurocholate The mixture was maintained at 37° C and samples were processed for electron microscopy after intervals of 10 30 and 60 minutes Each preparation was fixed in Karnovsky s fixative and embedded in cross linked

dure which included the use of the PGUA agar as primary cultivation medium (Fig 1) The results of the study show that the PGUA medium is highly reliable for the intended purpose None of 1,892 cultures identified as *E coli* on the basis of the PGUA reaction was incorrectly identified In contrast, the use of standard biochemical tests resulted in the misidentification of 1% of the isolates

Reliance on a single characteristic for bacterial identification is usually a hazardous principle However, this study demonstrated that the PGUA-reaction can be used with a high degree of confidence as a single differential test for *E coli* According to the procedure (Fig 1), PGUA negative *E coli* which constitute 5–6% of bacteria of this species are referred to identification by conventional means and are thus not misidentified Conversely, our data indicate that PGUA positive varieties of other enterobacteria, with the exception of shigellae, are non existing or are extremely rare This negates the problem of organisms being falsely identified as *E coli*

The highest frequency of β glucuronidase activity among shigellae was observed in the species *S sonnei* (98%) Since shigellae, except for *S sonnei* are lactose negative the concurrent use of the PGUA agar and MacConkey agar, which reveals the lactose fermentation reaction ensures that such strains are not misidentified as *E coli Shigella sonnei* is known to be a late lactose fermenter Although typically it does not change the indicator of the MacConkey agar within 24 hours incubation it may do so However shigella organisms are very rarely encountered in urine samples (2 3 this paper) for the processing of which the alternative procedure was designed If the method is applied in the examination of faecal samples it is advisable to include selected tests which would differentiate *Shigella* from *Escherichia* e g the indole and lysine decarboxylase tests

A considerable number of the *E coli* strains that lacked β glucuronidase activity belonged to one particular serotype i e 01 Kl The fact that the frequency of this serotype was significantly higher than it is in a non selected strain material (3) may indicate that the lack of this enzyme is a characteristic of a particular subpopulation of the species

The advantages in using the described alternative procedure are manifold The result of the examination of approximately half of all the urine samples i e samples that contained *E coli* as the sole organism, could be reported already after 18–24 hours following the reading of the initial plates In comparison the processing of samples by the conventional procedure required two to three days In order to take full advantage of this early identification, the problems of carrying out antibiotic susceptibility testing on primary plates have to be solved

Since it was not necessary to carry out standard identification on 94% of all *E coli* cultures the alternative method resulted in a considerable saving in media and time In spite of the relatively high expense of the chromogenic substrate used in the PGUA medium, the total percentage saving in media required for primary cultivation and identification of enterobacteria amounted to 46% as compared to the standard procedure Both this and the calculated saving in time (67%) may have considerable economic implications in routine bacteriological laboratories

We thank *Lena Poulsen* for excellent technical assistance Dr *H Lautrop* provided helpful suggestions and encouragement during the planning of this study Drs *Ida Orskov* and *Frits Orskov* are thanked for the serological examination of selected strains and Dr *Karen Lehbaek* was helpful in designing the PGUA medium The project was supported by the *Danish Medical Research Council* Grant No *512 8081*

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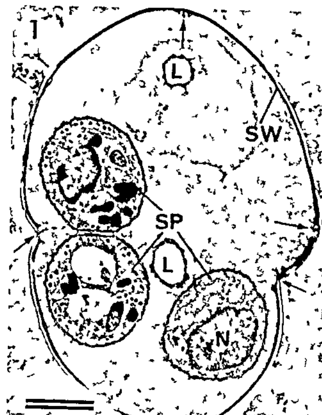
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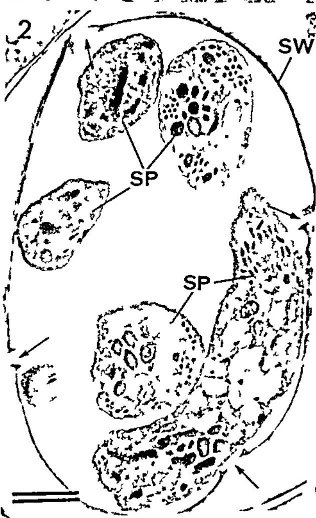
MATERIALS AND METHODS

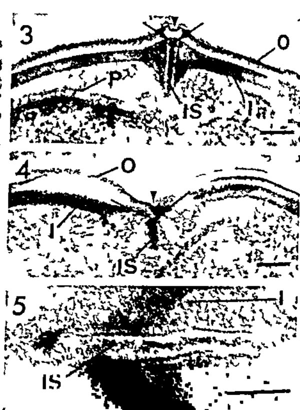
Oocysts were concentrated from the faeces of specific pathogen free cats which had been infected by feeding mouse brains containing tissue cysts of the SSI/119 strain of *T gondii* The oocysts were suspended in 2% potassium dichromate and allowed to sporulate at 27° C for 7 days prior to storage at 4° C The oocysts used in this study were approximately 2 months old After removal of the potassium dichromate the oocysts were treated with 6% sodium hypochlorite for 15 minutes, washed in water and then suspended in phosphate buffered saline (PBS) pH 7 3 At this stage the oocysts were ground to rupture the oocyst wall The excysting medium contained trypsin and sodium taurocholate dissolved in PBS in such amounts that the final concentration in the oocyst suspension was 0 25% (w/v) trypsin and 0 75% (w/v) sodium taurocholate The mixture was maintained at 37° C and samples were processed for electron microscopy after intervals of 10 30 and 60 minutes Each preparation was fixed in Karnovsky s fixative and embedded in cross linked

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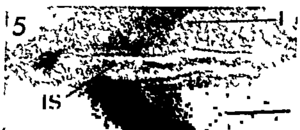
* Danish Medical Research Council Fellow Present address Department of Pathology University of Edinburgh Medical School Teviot Place Edinburgh Scotland

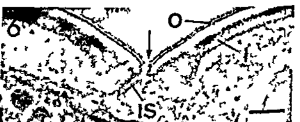

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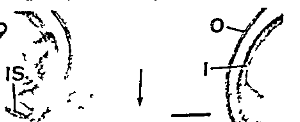

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Figures 1–17 are electron micrographs illustrating the changes which occur during the excystation of the sporozoites of *T gondii*

A double bar ( ) on a micrograph represents 1 µm and a single bar (–) represents 100 nm

The following abbreviations are used throughout I = inner layer of the sporocyst wall IS = interposing strip L – lipid N = nucleus O – outer layer of the sporocyst wall P – plasmalemma SP = sporozoite SW = sporocyst wall

Fig 1 A section through a sporocyst at an early stage of excystation The junctions of the plates forming the inner layer of the sporocyst wall can be seen (arrows) Note the beginning of an inward curling at the two lower junctions × 15 000

Fig 2 A longitudinal section through a sporocyst showing the sporocyst wall to be divided into four portions by breaks in the sporocyst wall at the junctions between the plates of the inner wall layer (arrows) × 15 000

Fig 3 A cross section showing the structure of the junction between plates of the inner layer prior to treatment with excysting medium The interposing strip and the thin band of osmiophilic material (arrows) joining the plates to the interposing strip can be seen Note the depression in the outer layer (arrow head) directly over the junction × 90 000

Fig 4 A cross section showing the structure of the junction after treatment with excysting medium The inward curling of the plates of the inner layer of the sporocyst wall has caused a separation of the plates at the base of the junction although they are still attached to the interposing strip (arrows) Note the inward bending of the outer layer (arrow head) × 90 000



Fig 6 A cross section showing the separation of the plates of the inner layer of the sporocyst wall Note that the interposing strip has remained attached to the margin of one plate and that the outer layer has bent inward *directly above the break (arrow)* × *90 000*

Fig 7 A similar section to that shown in Fig 6 except that in this case the interposing strip has disappeared The bend in the outer wall is also visible (arrow) × 90 000

Fig 8 A cross section of a sporocyst wall at a late stage in the excystation process A break is present in the outer layer (arrow) directly above the point of separation of the plates of the inner layer × 90 000

Fig 9 In this section the curling of the plates of the inner layer has caused the portions of the sporocyst wall to move apart forming a gap between the plates (arrow) Note the interposing strip attached to the margin of one plate × 90 000

bovine serum albumin The methods of post fixation embedding and examination were as described previously (6) The results are based on the examination of approximately 250 electron micrographs

RESULTS

After grinding the mixture containing both intact and ruptured oocysts was suspended in the excysting medium and examined by light microscopy It was found that sporocysts within intact oocysts did not undergo excystation even after treatment for more than 1 hour with the medium However excystation did occur in cases where the oocyst wall was ruptured resulting in the sporocysts being exposed to the medium

At the ultrastructural level it was observed that only sporocysts which had started to excyst were properly fixed and embedded The fine structure of the sporocyst wall prior to excystation has been described previously (7) It consists of a thin outer layer and an inner layer comprising four curved plates The structure of the junction between the plates prior to treatment with excysting medium is shown in Fig 3 and a detailed description of this region of the sporocyst wall has been given in a previous paper (7) After exposure to the excysting medium the first change observed was an inward curling at the periphery of the plates of the inner layer of the sporocyst wall (Fig 1) At the same time changes were also seen in the specialized junctions between plates (cf *Figs 3 & 4*) It appeared that the inner margins of the plates and the interposing strip had moved apart although they still remained attached at the exterior margin of the junction (Fig 4) In certain cases the interposing strip appeared to undergo some degeneration and presented an indistinct outline (Fig 5)

The curling of the plates was followed by an inward bending of the outer layer (Fig 4) This bending occurred at the shallow depressions which lay immediately above the *junctions of the plates of* the inner layer (Figs 4 6 & 7) It appeared that this inward curling of the plates continued and that the junction between them ultimately was broken (Figs 6 & 7) The interposing strip which was present at the junctions may have disappeared by this time (Fig 7) alternatively it may remain attached to the edge of one plate (Figs 6 & 9) The continued curling of the plates eventually caused the outer layer to rupture at the shallow depression where the bending commen[illegible]

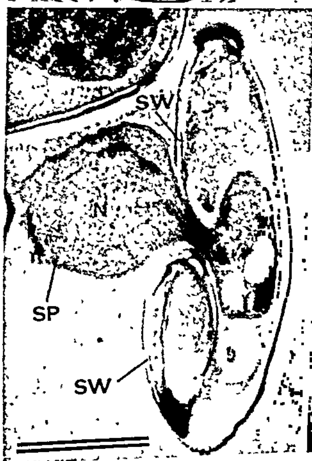
[illegible] layer (Figs 9 & 11) As this inward curling continued the portions of the



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Fig 10 A longitudinal section through a sporocyst Two of the junctions between the plates of the inner layer are still intact (small arrows) while at the other two junctions the plates have curled inward to produce an opening in the sporocyst wall (large arrows) ×15 000

Fig 11 Part of a section through a sporocyst showing the inward curling at the periphery of the separated parts of the sporocyst wall (arrows) Note that each portion consists of a plate of the inner layer and the overlying part of the outer layer ×45 000

Fig 12 This field of view shows a section through a sporozoite which has been deformed by the inward curling of a portion of the sporocyst wall ×30 000

Fig 13 A tangential section of the inner layer of the sporocyst wall after excystation showing the gap between two plates (large arrow) Note the fine striations (small arrows) and coarse striations (arrow heads) ×160 000

sporocyst wall were drawn apart (Figs 9 10 & 11) and the sporozoites could now escape through the openings thus created In a few sporocysts it was found that the junctions did not break simultaneously (Fig 10) In the majority of cases it was observed that the residual body present in the sporocyst (8) ruptured during excystation and a number of free lipid globules could be seen within the sporocyst (Figs 1 & 11)

Throughout the entire excystation process the two layers of the sporocyst wall remained separated by a space of approximately 20 nm The individual portions of the sporocyst wall continued to curl (Fig 11) until they finally formed tightly wound whorls (Figs 14 & 15) This process was so forceful that any sporozoite trapped within the curled plates was distorted (Fig 12)

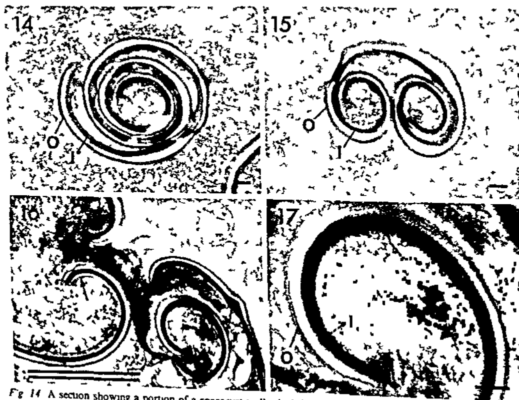


Fig 14 A section showing a portion of a sporocyst wall which has curled up to form a tight whorl ×45 000

Fig 15 This field of view shows a section of a portion of the sporocyst wall in which the curling has occurred from both margins ×45 000

Fig 16 Part of a section through a sporocyst which has been mechanically damaged Note that the various portions of the sporocyst wall have curled up (arrows) ×30 000

Fig 17 A higher magnification of Fig 16 which shows more clearly that curling of the sporocyst wall after mechanical damage involves both layers ×90 000

Two types of striations were observed on the plates of the inner layer of the sporocyst wall (7) These striations were also evident in the plates after the process of excystation was completed (Fig 13)

DISCUSSION

Intact oocysts of *T gondii* did not undergo excystation From this it would appear that the excysting fluid is only active on the sporocyst wall, but the stimulus which must occur *in vivo* to cause the rupture of the oocyst wall is unknown

The excystation of the sporozoites of a number of *Isospora* spp has been studied *in vitro* and it has been found that the structure of the sporocyst wall is related to the process of excystation Two distinct processes appear to be involved Firstly, sporozoites can escape via an aperture in the sporocyst wall resulting from the unplugging of the Stieda body (2, 11) This mechanism is similar to that reported for the closely related *Eimeria* spp (1, 3, 9, 10) The second mechanism operates where there is no Stieda body, the sporocyst wall collapses and by fragmentation into a number of portions permits sporozoite escape (4, 5, 12, 13)

The process of excystation in *T gondii* as demonstrated in the present study, clearly belongs to the second category The fine structure of the sporocyst wall is similar to that described for *I canis* and *I endocallimici* (12, 13) In a light microscope study of the excystation of *I arctopitheci* (5) folds and fissures on the sporocyst wall were observed These structures probably represent an inward curling and separation of the plates of the inner layer of the sporocyst wall in a manner similar to that presented in this study of *T gondii* The continued curling of separate portions of the sporocyst wall is similar to that observed for other species showing the second type of excystation process (4 5 12 13)

From our observations on the excystation of *T gondii* it would appear that the process can occur by one of two mechanisms The first possibility is that the excysting medium acts directly on the joints between plates of the inner layer of the sporocyst wall and digests the material attaching the plates to the interposing strip On the assumption that an inherent tension is present within the plates these would tend to curl inward and thereby eventually produce the breakage in the outer sporocyst wall layer We do have evidence that there is some inherent tension in the sporocyst wall It was observed on material from our sporulation studies (7, 8) that after a mechanical destruction of the sporocyst the portions of the sporocyst wall did curl inwards (Figs 16 and 17) This mechanism i similar to that proposed for the excystation of *I endocallimici* (13) A second possible mechanism fo the process of excystation is that the mediun triggers off reactions in the interior of the sporocys which somehow create the tension in the plates tha eventually causes them to curl inward With thi type of mechanism the separation of plates may b purely mechanical due to the forces exerted on th junction by the curling of the plates The actua curling must be very forceful as evidenced by th distortion of any trapped sporozoites and by th fact that it continues until tight whorls are formed Whether the excystation process actually occurs b one or other of the suggested mechanisms cannot b decided, but it should not be overlooked that it ma very well take place by a combination of the two

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Acta path microbiol scand Sect B *87* 285–290 1979

CHEMOTAXIS OR MIGRATION INHIBITION OF RABBIT PERITONEAL POLYMORPHONUCLEAR LEUKOCYTES CAUSED BY CHEMOATTRACTANTS AT VARIOUS CONCENTRATIONS

KJELL SVEEN

The Gade Institute Department of Microbiology Laboratory for Oral Microbiology University of Bergen Bergen Norway

Sveen K Chemotaxis or migration inhibition of rabbit peritoneal polymorphonuclear leukocytes caused by chemoattractants at various concentrations Acta path microbiol scand Sect B *87* 285–290 1979

Purified lipopolysaccharide (LPS) from *Veillonella* incubated in normal rabbit serum was tested for chemotactic activity on rabbit polymorphonuclear leukocytes (PMNs) in modified Boyden chambers In doses above those giving optimal response (over-optimal dose) a decrease of the PMN migration activity was found This decrease also correlated well with an increase in the migration inhibition of the PMNs as demonstrated with the capillary tube assay The PMN chemotactic factor isolated from LPS induced inflammatory exudate (LPS CF) in rabbits produced both a decrease *in chemotactic* response and a migration inhib tion of PMNs in over-optimal doses This inhibitory effect was not due to cytotoxicity proved by the trypan blue exclusion test Also a reduced locomotion of PMNs first preincubated with chemoattractants and then reactivated was shown when the *same* PMNs were restimulated to migration using the same chemoattractants This was interpreted as a deactivation of the cells A cross-deactivation was demonstrated between LPS-CF and casein The results from the experiments reported show that the Boyden chamber *may be used to* discriminate directional chemotaxis and migration inhibition It may also be concluded from the study that the reduced migration activity of PMNs at over-optimal doses of chemoattractants is not due to cytotoxicity but most probably is caused by a deactivation of the cells

Key words Leukochemotaxis migration inhibition lipopolysaccharide chemotactic factor casein

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Received 3 iv 79 Accepted 29 iv 79

Polymorphonuclear leukocytes are essential in the host response to acute infections The attraction of PMNs to the inflammatory area and the trapping of PMNs in the inflammatory focus are of importance in the inflammatory process (11) *It has* been suggested that these two events are effected either by the same molecule (4) or by separate molecular entities (17) In the immune response both chemotactic factors and factors inhibiting the migration (MIF) of PMNs *are released from* B and T lymphocytes stimulated by antigen In this situation chemotaxis and migration inhibition are bound to different molecular entities (17) Leukotactic factors are also generated by activation of *complement e g* by bacterial lipopolysaccharide (LPS) and [illegible] cytota[illegible] able to [illegible]

In [illegible] experiments concerning the leukotactic effect of different cytotaxins or cytotaxigens have shown that there is a dose response relationship *up to* a certain optimal dose of the chemoattractant The use of higher concentrations regularly leads to a decrease in number of PMNs accumulated (4 7 9)

Acta path microbiol scand Sect. B 87 285–290 1979

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In vitro (9) and *in vivo* (10 14) experiments concerning the leukotactic effect of different cytotaxins or cytotaxigens have shown that there is a dose response relationship up to a certain optimal dose of the chemoattractant The use of higher concentrations regularly leads to a decrease in number of PMNs accumulated (4 7, 9)

Viability Test

LPS Ve9 in equivalent amounts to that used for the testing of chemotactic activity was incubated in the same volume of serum LPS-CF was prepared in two-fold dilutions with Gey s medium as used in the chemotaxis assay To each dilution of LPS-CF approximately 1.2×10^7 PMNs were added before incubation at 37°C for 3 h The PMNs were thereafter isolated by centrifugation at 4°C and washed 3 times in cooled (4°C) Gey s medium The cells were tested for viability by the trypan blue exclusion test.

Chemotactic Stimulation of Preincubated Leukocytes

After being incubated for 3 h with different concentrations of LPS-CF the PMNs were washed 3 times in cooled Gey s medium thereafter suspended in this medium and kept at 1°C for 7 h for reactivation The cells were then washed 3 times in cooled Gey s medium suspended in Gey s medium containing 2 per cent BSA and adjusted to a concentration of 10^7 leukocytes per ml Each sample was tested for the capacity of migration against the same concentration of cytotaxin as it had been preincubated with using the Boyden chamber for measuring the chemotactic activity Another experiment was performed where PMNs after being preincubated with d ffe ...

medium containing 2 per cent BSA and stimulated by 1 per cent casein to migration activity in the Boyden chamber In add t on ...

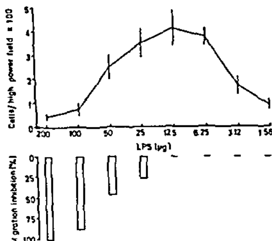


Fig 1 Upper panel Leukocyte chemotaxis induced in Boyden chambers by LPS-Ve9 incubated in normal rabbit serum diluted 1 1 with Gey s medium at 37°C for 30 min and at *56°C* for 30 min Each point represents the mean cell count ± standard deviation (vertical bars) for triplicate chambers (solid curve) Lower panel migration inhibition of leukocytes induced by LPS-Ve9 incubated as described above with serum diluted 1 1 with M 199 FCS measured with the capillary tube test Bars represent means of migration inhibition from six capillaries Values of more than 25 per cent were considered as significant migration inhibition with reference to LPS free petri dishes

EXPERIMENTS AND RESULTS

The migration activity of rabbit PMNs in response to different doses of LPS-Ve9 and LPS-CF was examined in Boyden chambers and by the capillary tube test in parallel experiments (Figs 1 and 2) Maximal leukocyte chemotaxis was achieved by 12 5 µg of LPS The regression in attraction activity at higher doses of LPS Ve 9 was parallelled by a steady rise in migration inhibition (Fig 1) Essentially the same results were obtained using LPS-CF as chemoattractant (Fig 2)

The results obtained when the PMNs were pre exposed to different concentrations of LPS in serum ...

... reduced compared to that obtained without preincubation with chemoattractant (cf Fig 1) Leukocytes

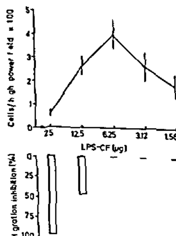


Fig 2 Upper panel Leukocyte chemotaxis induced in Boyden chambers by LPS-CF in Gey s medium Each point represents the mean cell count ± standard deviation (vert ... curve) Low ... induced by ... capillary tu ... Bars represent means of migration inhib t on fro ...

The present study was undertaken to examine if there is a correlation between decreased chemotaxis and inhibition of leukocyte migration if both activities can be induced by the same molecular entity, and if the impaired chemotaxis observed at over optimal doses of a chemoattractant is due to a cytotoxic effect

To obtain information on the interplay between PMNs and chemoattractants experiments were included where PMNs stimulated with cytotaxin or cytotaxigen were allowed to regain their capacity to respond *i e* to become reactivated before a repeated stimulation with a chemoattractant was carried out

MATERIALS AND METHODS

Rabbit Polymorphonuclear Leukocytes (PMNs)

PMNs were obtained from the peritoneal cavity of adult New Zealand White rabbits and isolated as previously described (9) Before being tested for migration in modified Boyden chambers (6) (Neuroprobe Bethesda Md USA) the PMNs were suspended in Gey s medium containing antibiotics (50 µg Garamycin® (Schering Corporation Bloomfield N J) and 2 5 µg Fungizone® (Squibb Flow Laboratories Irvine Scotland) per ml) and adjusted to a 2 per cent solution with bovine serum albumin (BSA) (Armour Pharmaceutical Company Ltd Eastbourne England) giving a final cell count of approximately 10^7 cells per ml For the migration inhibition test (see below) Medium 199 containing 5 per cent (v/v) inactivated foetal calf serum (M 199 FCS) (Flow Laboratories) and the same concentration of antibiotics as added to Gey s medium was used Cells from the batch used for chemotactic assays were suspended at a concentration of 30×10^6 cells per ml in the medium

Source of Complement

Pooled sera from normal rabbits (NRS) containing 0 1 per cent merthiolate (w/v) were stored at −25°C until used

Chemoattractants

Cytotaxigen Bacterial lipopolysaccharide (LPS) was isolated from *Veillonella parvula* strain Ve9 by the [illegible] (8) and [illegible] as

one min (MSE/Mullard 60W 20 kc/s)

Cytotaxin The isolation of LPS from *Bacteroides fragilis* subspecies *fragilis* strain Lille E 323 (8) the implantation on rabbits of Teflon® chambers (14) the induction of inflammatory exudate and chemotactic factor generation (LPS CF) by LPS and the isolation of LPS CF (12) were carried out as previously described Casein was obtained from E Merck AG Darmstadt W Germany

Measurement of Chemotactic Activity

The two compartments of each modified Boyden chamber were separated by a Millipore filter (Millipore Filter Corp Bedford Mass USA) with a pore size of 3 µ The lower compartment having a volume of 0 2 ml was filled with chemoattractant The different chemoattractants were prepared either from preincubation of LPS Ve9 in a two fold dilution ranging from 200 to 1 56 µg in 0 1 ml of NRS or LPS-CF in 0 2 ml of Gey s medium in a two fold dilution ranging from 25 to 1 56 µg Casein was used in a one per cent solution Before placing the filter discs in the chambers 0 3 ml of the cell suspension *i e* 3×10^6 PMNs was spun down on the filter as described previously (9) The upper compartment was filled with Gey s medium containing antibiotics and BSA and the chambers placed in a humid incubator at 37°C for 3 h After the incubation period the filters were stained cleared and mounted on glass slides (9) The number of PMNs on the distal side of the filter disc was calculated as described before (9) and the chemotactic activity expressed as the number of PMNs per high power field

Migration Inhibition Test

PMNs were transferred by means of vacuum suction to heparinized (A/S Apothekernes Laboratorium for Specialpræparater Oslo) microhaematocrit capillary tubes (capacity approx 75 µl) (Vitex Denmark) which prior to filling were sealed at one end by melting the glass (3) The capillary tubes were centrifuged at 900 rev/min for 5 min and cut over just beneath the cell fluid interphase The cell-containing portion was immediately placed in small petri dishes (4 5 cm diameter) (Sterilin Ltd England) containing 4 ml of the medium or the control In each petri dish 3 capillary tubes were held in place with a small amount of silicone Each dilution of either LPS serum incubation mixture or LPS CF suspension was tested in 2 petri dishes Two petri dishes containing only M 199 FCS served as controls LPS Ve9 which was preincubated in 0 1 ml serum (37°C for 30 min and 56°C for 30 min) was tested in two fold dilutions ranging from 200 to 1 56 µg and LPS CF was tested in two fold dilutions ranging from 25 to 1 56 µg per 0 2 ml of M 199 FCS Before dilution with M 199 FCS the preincubated LPS serum mixtures were centrifuged at 28 000 × *g* at 4°C for 30 min (IEC Universal Model UV) and the supernatants pipetted off After incubation at 37°C in humid air containing 5 per cent CO_2 for 6 h the petri dishes were placed under an inversion microscope and photographed by a Polaroid camera using a four fold magnification objective The area of migration was calculated by planimetry and the percentage migration as

$$\frac{\text{migration area with antigen}}{\text{migration area without antigen}} \times 100 \; (3)$$

The average area of migration for each dilution of test substance was calculated from 6 capillary tubes and the percentage of migration inhibition was calculated from the average area of the control

PMNs being preincubated in Gey s medium contai ning LPS only (cf Figs 3 and 4) indicates that the decreased migration activity from PMNs preincuba ted in increasing doses of serum-derived cytotaxin may be due to a binding of cytotaxin molecules to the receptor sites on their membranes This binding may result in either a partial or complete loss of an esterase activity The receptor sites may not be reactivated or renewed or an exhaustion of the metabolic processes and/or an increase of the surface adhesiveness may thus be important factors in the decreased migration activity In addition the demonstration of a decreased chemotactic response paralleling a migration inhibition from over-optimal doses of LPS-CF indicates that these events are not due to chemotactic inactivators or inhibitors in the serum alone The possibility has also been suggested that the loss of migration function of human neutrophils pre-exposed to high concentration of chemotactic agents may be *attributable to the* interaction of the cells with reactive forms of oxygen deriving from the cytotaxin induced burst of oxidative metabolic activity (5)

Cross-deactivation of the cytotaxins C5a and kallikrein (2) and between different complement derived chemotactic factors (15) have been repor ted The results of this study also indicate that the serum-derived cytotaxin LPS-CF and casein may have common receptor sites on the PMN mem *brane*

It is possible that a chemotactic gradient of high concentration across the filter may activate mem brane receptors in a concentric distribution all over the cell surface thus leading to a trapping of the cells This might represent an important mechanism in the control of inflammatory responses mediated by leukotactic factors

Jungi (4) suggested a cell surface adhesiveness *in vitro* either to each other or to the filter or plastic material as a factor for migration inhibition *In vivo* decreased PMN accumulation is also observed using relatively high doses of LPS (10) *Therefore* the adhesiveness to foreign materials *can be ruled* out as the major cause Nevertheless an accumula tion of leukocytes in small vessels near to the source

The involvement of the recognition system of the cells might thus impaire the cell sensitivity for the diffusion gradient Furthermore it must be remem bered that *the* migration inhibition activity on PMNs by high concentrations of

due to th

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release of such factors has been reported to take place when rabbit peritoneal PMNs are phagocyto sing (1) However, these inactivators or inhibitory factors are not identical to the migration inhibition factor (MIF) produced by antigen stimulated B or T lymphocytes

Taken together the results from the experiments reported show that the Boyden chamber may be used to discriminate directional chemotaxis and migration inhibition as has also been suggested by others (4 19) Thus the reduced migration activity of PMNs found at over-optimal doses of chemoat tractants is not due to a cytotoxicity but merely to a deactivation of the cells most probably contributed by several non-specific components

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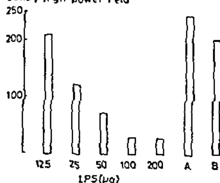


Fig 3 Migration of rabbit peritoneal leukocytes measured in Boyden chambers after the leukocytes were preincubated for 3 h at 37°C in chemotactic medium (various amounts of LPS Ve9 in normal rabbit serum prepared as in Fig 1) After reactivation (washed 3 times and then resuspended in Gey s medium for 7 h at 1°C) the leukocytes were stimulated to migration against the same chemotactic medium as they were preincubated with Bar A represents leukocytes first preincubated with 12 5 μg of LPS Ve9 in 0 2 ml of Gey s medium reactivated and then stimulated to migration against 12 5 μg of LPS Ve9 in serum (chemotactic medium prepared as in Fig 1) Bar B represents leukocytes preincubated with heat inactivated rabbit serum The leukocytes were reactivated and then stimulated to migration as in A Each bar represents the mean cell count from duplicate chambers

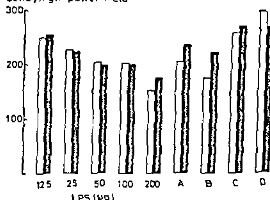


Fig 4 Migration of rabbit peritoneal leukocytes measured in Boyden chambers after being preincubated *for 3 h* at 37°C in medium containing various concentrations of LPS Ve9 in rabbit serum (prepared as in Fig 1) or with A 6 25 μg and B 12 5 μg of LPS CF in 0 2 ml of Gey s medium Bar C represents leukocytes ... inactivated rabbit serum alone and ... Gey s ... Fig 3) for 12 h at 1°C migration (open columns) was measured in response to 12 5 μg of LPS Ve9 incubated in rabbit serum (prepared as in Fig 1) or to a 1 per cent casein solution (cross hatched columns) Each bar represents the mean cell count from duplicate chambers

preincubated in Gey s medium containing 12 5 μg of LPS in 0 2 ml showed higher migration activity than cells preincubated only in serum Before the PMNs were reactivated, trypan blue exclusion was 95 per cent compared to 92 per cent after the reactivation

Leukocytes *first preincubated with different* concentrations of LPS in serum and thereafter reactivated for 12 h at 1°C showed a decreased migration activity when restimulated by 12 5 μg of LPS (cf Fig 4) The reduced chemotactic response of the PMNs was most significant when high doses of LPS were used in the preincubation mixture When stimulated against a one per cent casein solution the PMNs showed a response pattern similar to that seen when stimulated against LPS Cells preincubated either in inactivated NRS or Gey s medium containing LPS showed the highest migration activity About 90 per cent of the leukocytes excluded the trypan blue dye after 12 h of reactivation No difference in the percentage of cells taking up trypan blue dye was found either when over-optimal doses of cytotaxin in Gey s medium or when Gey s medium alone was used in the preincubation period

DISCUSSION

These studies demonstrate that the inhibition of the *migration in vitro* of rabbit peritoneal PMNs induced by high concentrations of the cytotaxigen LPS Ve 9 or the cytotaxin LPS CF was paralleled by a decrease in chemotaxis (cf Figs 1 and 2) These results indicate that the same recognition mechanism is involved both in inhibition of directional chemotaxis and in *migrational inhibi*tion depending on the concentration The detachment of cells from the chemotactic side of the filter has been ruled out as a major factor in the decrease in cell accumulation at over-optimal doses of attractants (9) The results of the experiments reported here also exclude cytotoxicity as an explanation of this phenomenon

PMNs preexposed to various concentrations of LPS CF results in a suppression of the same PMNs from migration towards the same chemotactic gradient the suppression being proportional to the dose of cytotaxin used in the preincubation (13) Such a phenomenon has been termed »deactivation« (16) The migration activity of PMNs after reactivation (cf Fig 3) was *considerably decreased* compared to that obtained when unstimulated cells were used (cf Fig 1) The different ages of the cells may be a cause of their different chemotactic responses However the higher response from

ANTIBODIES TO THE STRAIN-SPECIFIC AND CROSS-REACTIVE DETERMINANTS OF THE HAEMAGGLUTININ OF INFLUENZA H3N2 VIRUSES

I Preparation of *Antibodies and* in Vitro *Studies*

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Haaheim L R & Schild G C Antibodies to the strain-specific and cross reactive *determinants* of the haemagglutinin of influenza H3N2 viruses I Preparation of antibodies and *in vitro* studies Acta path microbiol scand Sect B *87* 291–299 1979

... were obtained which are designated strain specific and cross reactive and characterized in terms of their antigenic specificities for HA ...

... involving ... of different antibody preparations in the same wells in immunoplates containing intact virus particles The cross reactive and *strain specific* antibodies differed in their property of mutual interference of attachment to antigen The *results* ...

... per μg IgG than the cross reactive antibodies

Key words Influenza haemagglutinin strain specific antibodies cross reactive antibodies antigenic sites

L R Haaheim National Institute of Public Health Vaccine Department Postuttak Oslo 1 Norway

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The two surface antigens of human influenza A virus the haemagglutinin (HA) and the neuraminidase (NA) undergo infrequent major antigenic changes (shift) associated with the appearance of influenza pandemics In interpandemic periods more frequent minor changes (drift) occur resulting in the progressive modification of the surface antigens of viruses of successive prevalence The mechanism of antigenic 'drift' is that of selection of antigenic mutants of viruses ...

Acta path microbiol scand Sect B *85* 374–380, 1977
10 *Sveen, K* Rabbit polymorphonuclear leukocyte migration *in vivo* in response to lipopolysaccharides from *Bacteroides, Fusobacterium* and *Veillonella* Acta path microbiol scand Sect B *85* 381–387, 1977
11 *Sveen, K* The importance of C5 and the role of the alternative complement pathway in leukocyte chemotaxis induced *in vivo* and *in vitro* by *Bacteroides fragilis* lipopolysaccharide Acta path microbiol scand Sect B *86* 93–100, 1978
12 *Sveen, K* Rabbit polymorphonuclear leukocyte chemotactic factor generated *in vivo* by *Bacteroides fragilis* lipopolysaccharide I Isolation and physicochemical characterization Acta path microbiol scand Sect B *86* 229–236, 1978
13 *Sveen, K* Rabbit polymorphonuclear leukocyte chemotactic factor generated *in vivo* by *Bacteroides fragilis* lipopolysaccharide II Antigenic and biologic properties Acta path microbiol scand Sect B *86* 237–245, 1978
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with concentrates of B/HK virus in order to remove antibodies to the 'host antigen

Immuno-double-diffusion (IDD) and Single radial-diffusion (SRD) tests IDD tests were performed as described by *Schild* (11) using as antigen virus concentrates disrupted with sodium sarkosyl detergent (NL97 Ciba-Geigy GB) SRD tests were made according to a previously described method (12). For sequential application of antibody in the same SRD wells so-called SRD competition experiments the following method was employed 20 µl samples of antibody were introduced and left to diffuse for 24 hours Further samples of antibody were introduced and left to diffuse for another 24 hours before reading the final zone Before testing for mutual additiveness of their SRD reaction zones all antibody preparations were diluted so as to give approximately equal sized zones within the range of 18–22 mm² Complete additiveness of two antibody zones is taken as indicating that the antibody preparation first added prevents the attachment of the one added subsequently No additiveness means a lack of such interference

RESULTS

Antigenic Relationship between H3 Haemagglutinins

HI tests Table 1 shows the cross reactions in HI tests between different strains of the H3N2 subtype, using post infection ferret sera and immune goat sera to purified HA antigens In general the post infection ferret sera were more specific than the immune goat sera There was no detectable HI cross reaction between the chronologically *most* distant strains HK/68 and Vic/75, in tests with their corresponding post infection ferret sera However antisera to the intermediate variants Eng/72 and PC/73 reacted with all the test strains The relative strain specificity of the reactions of the post-infection ferret sera is in keeping with the finding that ferrets respond to an initial infection by producing antibodies which react predominantly strain-specifically in SRD tests (16)

The results of HI assays with immune goat sera, in contrast to those of the post infection ferret sera, showed broader cross reactivity between strains

IDD tests The immune anti-H3 sera used in the present study showed subtype specificities in IDD tests No precipitin lines were detected with influenza viruses of other HA antigen subtypes, including human H0N1, H1N1 and H2N2 viruses and avian 'N'/49 (Hav2Neq1) virus Fig 1a shows the IDD reactions of goat immune serum to purified HK/68 HA antigen tested against strains of the H3 H2 and Hav2 subtypes On the basis of the common precipitin line observed between H3 variants, these strains were shown to share one or more common antigenic determinants The pattern of spur formation indicates some determinants have changed completely during the evolution from HK/68 to Vic/75 Likewise, antiserum to purified Vic/75 HA antigen showed the converse pattern of antigenic relationship (Fig 1b)

Reactivity and Specificity of SS- and CR-Antibodies

IDD tests When antiserum to the HA antigen of HK/68 virus was absorbed with Vic/75 virus the *remaining antibodies* (SS'_{HK}) gave precipitin lines with all the test strains except Vic/75 (Fig 2a) A spur between HK/68 and Eng/72 viruses was also observed The antibodies eluted from the Vic/75 virus (CR'_{HK}) were cross reactive and reacted with all strains without spurs (Fig 2b)

After absorbing the anti HK/68 HA serum with the more closely related Eng/72 variant, a more highly strain specific population of antibodies (SS''_{HK}) remained which reacted with HK/68 virus only (Fig 3a) The CR-antibodies eluted from the

TABLE 1 *Antigenic Relationships between H3N2 Strains as Indicated by Cross reactions in HI Tests Performed with Post infection Ferret Sera and Hyperimmune Goat Anti HA Sera*

Virus strains	Antiserum to HK/68		Eng/72		PC/73		Vic/75	
	F[a]	G[b]	F	G	F	G	F	G
HK/68	3840	25600	2560	6400	640	12800	<10	4800
Eng/72	160	4800	5120	12800	2560	25600	80	6400
PC/73	30	1200	960	9600	1920	38400	240	9600
Vic/75	<10	300	320	5200	640	12800	1280	51200

[a] Post infection ferret serum

[b] Immune goat serum to purified HA antigens

presence of two types of antigenic determinants on the haemagglutinin molecule One type of determinant appeared to be antigenically common for the HA antigens of virus strains within a given subtype whilst other determinants were relatively strain specific Some of these latter determinants may change completely during drift Corresponding populations of antibody molecules directed against common and strain specific determinants have geen isolated and studied (5 18)

The main object of the present study was to investigate the serological properties of the strain specific (SS) and cross reactive (CR) antibodies *in vitro* in terms of their potency per µg IgG protein In order to determine the probable location of the two types of antigenic determinants on the HA molecule steric inhibition experiments using single radial diffusion tests were performed

MATERIALS AND METHODS

Virus Strains

The high yielding recombinant strains used were X31 MRC2 MRC11 and X47 posessing the surface antigens of the H3N2 variants A/Hong Kong/1/68 A/England/42/72 A/Port Chalmers/1/73 and A/Victoria/3/75 respectively These strains will be designated HK/68 Eng/72 PC/73 and Vic/75 throughout the text Other virus strains used were A/PR/8/34 (H0N1) A/USSR/90/77 (H1N1) A/Singapore/1/57 (H2N2) A/chicken/Germany/ N /49 (Hav2Neq1) and B/Hong Kong/8/73 These three latter strains will be designated Sing/57 N /49 and B/HK respectively All viruses were obtained from the collection of the National Institute for Biological Standards and Control London Viruses were propagated in 10-day old embryonated chick eggs and purified and concentrated as described by *Skehel & Schild* (17)

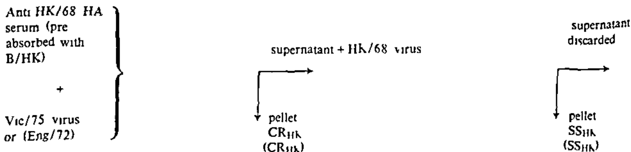
Sera

Post infection ferret sera Ferrets were infected intranasally with 10^6–10^7 egg infective-doses (50%) (EID_{50}) of virus and bled 12–14 days after infection

Immune anti HA sera Purified HA antigens were isolated from the H3N2 strains by use of the proteolytic enzyme bromelain (1) and potent immune sera to these antigens were raised in goats (15)

Preparation of Strain specific (SS) and Cross reactive (CR) Antibodies

Anti HA sera prepared in goats were absorbed with B/HK virus in order to remove antibodies to the 'host antigen (4) These sera were subsequently absorbed with strains of the H3N2 subtype using essentially the method described by *Laver et al* (5) For the preparation of CR antibodies concentrates of appropriate heterologous H3N2 viruses were added to the B/HK absorbed antisera The virus antibody complexes were then pelleted *in the ultracentrifuge and the antibodies* were eluted from the virus by resuspending the pellet in cold 0 1 M glycine HCl buffer pH 2 5 followed by brief ultrasonication After centrifugation to deposit the virus particles the supernatant containing the eluted CR antibodies was adjusted to neutrality by dialyzing overnight in the cold against phosphate buffered saline (PBS) pH 7 2 The serum supernatants containing the SS antibodies after absorption with the heterologous virus strains were always absorbed with the corresponding homologous virus variant and subjected to an identical centrifugation-elution procedure in order to obtain SS antibody preparations comparable with the eluted CR antibodies The flow diagram for the preparation of SS and CR antibodies was as follows



Similar procedures were followed for immune goat serum to purified Vic/75 HA but only absorption with the most distant strain HK/68 was carried out in the first absorption cycle These antibodies will be called SS_{Vic} and CR_{Vic} Assay of the IgG content in these antibody preparations was performed by the Mancini SRD (cf below) technique (7) using rabbit *anti goat IgG* serum (Cappel Laboratories Pa USA) in the agarose gel Goat IgG was used as reference antigen This was purified by DEAE chromatography essentially as described previously *(2) except that the anionic* exchanger DEAE-cellulose (DE 52 Whatman GB) in 0 015 M phosphate buffer pH 7 6 was used (8) Protein assays were performed by the method of *Lowry et al* (6)

Serolog cal Methods

Haemagglutinat on inhibit on (HI) tests These tests *were carried out by standard methods* (19) but in some experiments with immune anti HA goat sera the antibody and virus were left for 5 minutes at room temperature or 11 hours at 4°C before adding the red blood cells Before testing the goat sera were absorbed

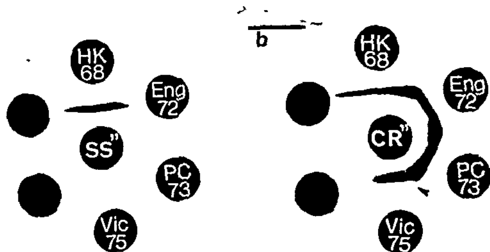


Fig 3 IDD reactions with SS_{HK} antibody (Fig 3a) and CR''_{HK} antibody (Fig 3b) obtained from immune goat serum to purified HK/68 HA antigen by absorption with Eng/72 virus (for experimental details see legend to Table 2 and Materials & Methods) The subscript »HK« is omitted in the figures Arrowhead indicates spur

strains more precise information concerning their specificity and potency was obtained (Table 2) It was clear that the two populations of SS antibodies (SS_{HK} and SS''_{HK}) had different properties The SS_{HK} antibodies gave smaller annulus areas with the virus variants Eng/72 and PC/73 and did not react with Vic/75 virus whereas the SS_{HK}-antibodies reacted only with the HK/68 variant

The two preparations of CR antibodies (CR'_{HK} and CR''_{HK}) gave clear SRD reactions with all the test strains It can be seen from Table 2 that the CR'_{HK}-antibodies were of approximately the same potency

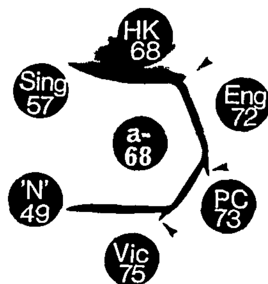
TABLE 2 *SRD Experiments Showing Different Specificity and Potency for SS- and CR antibody Preparations Obtained from Immune Goat Serum to HK/68 HA Antigen*

Virus in immunoplate	Serum absorbed with B/HK concentrate	Antibody preparations tested [a)]			
		SS_{HK}	CR_{HK}	SS''_{HK}	CR''_{HK}
HK/68	151 4[b)] (100)	140 3 (100)	28 2 (100)	110 2 (100)	70 8 (100)
Eng/72	94 4 (63)	101 5 (72)	24 8 (88)	–[c)]	56 8 (80)
PC/73	72 8 (48)	41 6 (30)	26 5 (94)	–	40 7 (57)
Vic/75	59 8 (39)	–	24 8 (88)	–	31 0 (44)

[a)] SS_{HK} represents residual (strain-specific) antibodies after absorption with Vic/75 virus whereas CR_{HK} represents cross reactive antibodies eluted from Vic/75 virus
SS''_{HK} represents residual (strain specific) antibodies after absorption with Eng/72 virus whereas CR''_{HK} represents cross reactive antibodies eluted from Eng/72 virus

[b)] Zone area (mm^2) of opalescent annulus Figures in brackets are percentage values of zone obtained in homologous immunoplate

[c)] No detectable zone



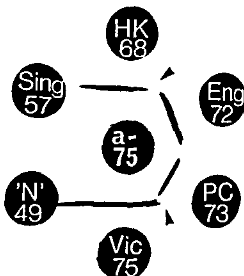
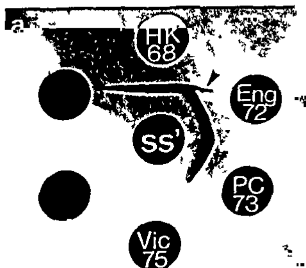
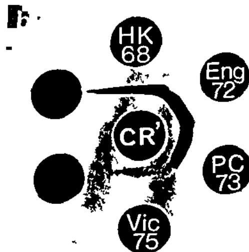


Fig 1 IDD reactions of immune goat serum to purified HA antigens of HK/68 and Vic/75 viruses for Fig 1a and Fig 1b, respectively The centre wells contain sera absorbed with B/HK virus in order to remove antibodies to the host antigen' The peripheral wells contain virus concentrates disrupted with the detergent sodium sarkosyl at a final concentration of 1% (v/v) The notations represent the H3N2 strains A/Hong Kong/1/68 A/England/42/72 A/Port Chalmers/1/73 and A/Victoria/3/75, respectively, and the strains A/chicken/Germany/ N /49 (Hav2Neq1) and A/Singapore/1/57 (H2N2) Spurs are indicated by arrowheads

Eng/72 strain (CR''_{HK}) gave precipitin lines with all the H3N2 viruses tested (Fig 3b) A spur could be seen between the PC/73 and Vic/75 viruses, which indicated that this preparation of CR-antibodies distinguished between the strains to some degree

SRD tests The above mentioned preparations of SS- and CR-antibodies were tested in different immunoplates containing equal amounts of Sing/57 and 'N'/49 viruses No zones of opalescence were observed (not shown), thus confirming the subtype specificity of the preparations found in IDD tests When equal volumes of these antibody preparations were tested in different immunoplates containing equal amounts of the various H3N2 test





2b) obtained from immune goat serum mental details see legend to Table 2 and whead indicates spur

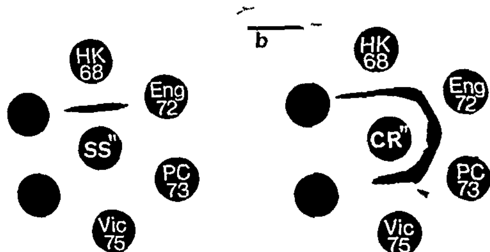


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[b]) Zone area (mm²) of opalescent annulus Figures in brackets are percentage values of zone obtained in homologous immunoplate

[c]) No detectable zone

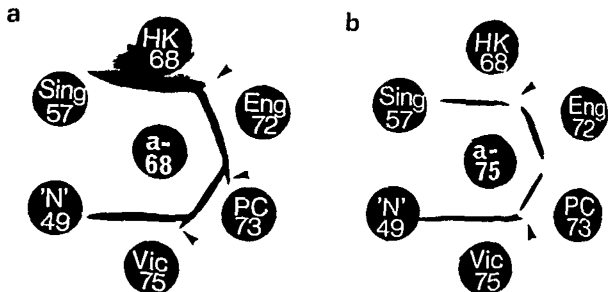
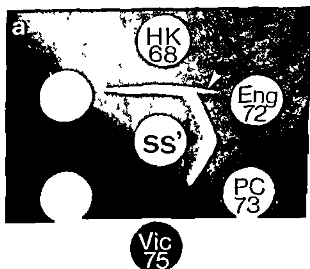
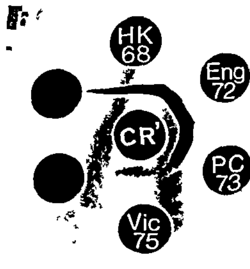


Fig 1 IDD reactions of immune goat serum to purified HA antigens of HK/68 and Vic/75 viruses for Fig 1a and Fig 1b, respectively The centre wells contain sera absorbed with B/HK virus in order to remove antibodies to the 'host-antigen' The peripheral wells contain virus concentrates disrupted with the detergent sodium sarkosyl at a final concentration of 1% (v/v) The notations represent the H3N2 strains A/Hong Kong/1/68, A/England/42/72, A/Port Chalmers/1/73 and A/Victoria/3/75, respectively, and the strains A/chicken/Germany/'N'/49 (Hav2Neq1) and A/Singapore/1/57 (H2N2) Spurs are indicated by arrowheads

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SRD tests The above-mentioned preparations of SS- and CR-antibodies were tested in different immunoplates containing equal amounts of Sing/57 and 'N'/49 viruses No zones of opalescence were observed (not shown), thus confirming the subtype specificity of the preparations found in IDD tests When equal volumes of these antibody preparations were tested in different immunoplates containing equal amounts of the various H3N2 test





... SS- ...antibody (Fig 2a) and CR_{HK}-antibody (Fig 2b) obtained from immune goat serum ... for experimental details see legend to Table 2 and ...es Arrowhead indicates spur

TABLE 4 *Specific Activities (SRD Zone Area or Titre/ µg IgG) of Antibody Preparations in Serological Tests with the Homologous Virus Strains*

Anti HA antibody against	Specificity[a])	Serological tests	
		SRD[b])	HI[c])
HK/68	SS_{HK}	36	33
	CR_{HK}	28	18
	SS_{HK}	46	41
	CR_{HK}	33	15
Vic/75	SS_{Vic}	56	41
	CR_{Vic}	32	6

a) See legend to Table 2 and Materials and Methods

b) Ratio × 10^3 between zone area (mm^2) in SRD and IgG concentration (µg/ml) data from Table 2

c) Ratio × 10 between HI titres (geometric mean of duplicate tests) and IgG concentration (µg/ml) data from Table 3

length of time allowed for virus and antibody to interact was demonstrated most clearly for the CR-antibodies

Specific activities of the SS- and CR antibodies The serological activities fo the antibody preparations were related to their IgG content in order to establish their specific activities in HI and SRD tests Table 4 shows the specific activities expressed as serological potency (titre or zone annulus area) per µg IgG protein The SS antibodies gave higher HI titres and larger SRD zones for the same amount of IgG than the CR antibodies

Interference between SS- and CR-Antibodies in SRD Tests

SRD competition experiments were performed involving sequential application of different anti body preparations in the same wells in immunoplates containing intact HK/68 virus It was found that the same antibody preparation applied twice to the same well did not always increase the zone size exactly twofold This was found to be dependant on the initial zone size and/or time allowed for diffusion If a SRD zone annulus was less than 25 mm^2, the degree of homologous addition was always found to be near 100% (*i e* twofold increase) when diffusion time was 24 hours All antibody preparations were therefore diluted before testing so as to give zone areas within the range of 18–22 mm^2 Table 5 shows the results of these experiments It was found that the CR antibodies when applied first, completely excluded the SS-antibodies in most instances since in that situation the zones were additive This was not the case when the SS antibodies were applied first, here the zones were significantly less additive Tests with SS_{HK}- and CR_{HK} antibodies showed intermediate patterns of additiveness

Similar experiments with SS_{Vic} and CR_{Vic} antibodies tested in immunoplates containing Vic/75 virus gave essentially similar results to those described for the anti HK/68 HA antibodies *i e* the CR antibodies excluded the binding of the SS antibodies when tested in that order

DISCUSSION

This report is an extension of earlier findings (5, 14) that the HA antigens of viruses belonging to the H3

TABLE 5 *SRD Experiments Investigating Degree of Additiveness of Zones Produced by Antibody Preparations of Different Specificity from Immune Goat Serum to HK/68 HA Antigen by Sequential Application of Antibody*

Antibody preparations tested		Percentage additiveness			
		Homologous		Heterologous	
		(SS + SS)	(CR + CR)	(CR + SS)	(SS + CR)
SS_{HK}	CR_{HK}	97 (104 92)[a])	100 (106 94)	101 (109 94)	47 (51 44)
SS_{HK}	CR_{HK}	95 (98 92)	98 (104 93)	80 (86 72)	66 (74 58)
SS_{HK}	CR_{HK}	93 (101 90)	93 (97 90)	100 (104 95)	55 (62 47)
SS_{HK}	CR_{HK}	95 (103 91)	95 (101 91)	97 (103 90)	42 (49 36)

a) All entries are mean of 5 [illegible]s with highest and lowest scores in brackets

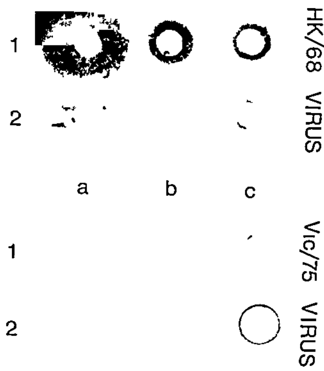


Fig 4 Specificity and potency of cross reactive and strain specific antibody preparations in SRD immunoplates containing HK/68 and Vic/75 virus strains Upper panel HK/68 virus in immunoplate Lower panel Vic/75 virus in immunoplate The same six antibody samples were tested in both plates On row 1 left to right in both panels the antibody samples were a) Goat anti HK/68 HA immune serum absorbed with B/HK virus b) SS_{HK} antibody and c) CR_{HK} antibody On row 2 left to right in both panels the antibody samples were a) Goat anti Vic/75 HA immune serum absorbed with B/HK virus b) SS_{Vic}-antibody and c) CR_{Vic} antibody For more detailed description of the antibody samples see legend to Table 2 and Materials & Methods

when tested against all the H3N2 strains in question as indicated by similar sized zone areas in immunoplates containing the various H3N2 antigens This suggests that they were directed essentially against determinants which were common for all the H3N2 strains used in this study On the other hand the CR_{HK} antibodies exhibited some degree of specificity for the homologous variant as the zone areas decreased when tested against progressively more distant strains

Antiserum to purified HA antigens of Vic/75 virus was absorbed with the HK/68 variant leaving in the supernatant a population of antibodies designated SS_{Vic} The eluted antibodies from the HK/68 virus were called CR_{Vic} These two preparations of antibodies together with the SS_{HK} and CR_{HK} antibodies were tested in immunoplates containing HK/68 and Vic/75 viruses The results of these experiments are shown in Fig 4 In can be seen that the SS antibodies reacted only with the homologous viruses whereas the CR antibodies gave SRD reactions with both strains

HI experiments In conventional HI tests which allowed the virus and antibody to interact for a short time before adding the erythrocytes the CR antibodies were found to react poorly with the most distant strains (Table 3) despite the positive reactions observed in IDD and SRD tests However if the virus antibody mixture was incubated for a long period of time (11 hours at 4°C) the CR antibodies gave markedly higher titres Although the specificity of the antibody preparations in HI tests agreed generally with the results obtained by SRD the CR antibodies reacted to higher HI titres with the homologous than with the heterologous strains Also the relative increase in HI titres with

TABLE 3 *Effect on HI Titres with Length of Interaction Time for Virus Antibody Mixtures before Adding Chick Erythrocytes Tests with SS and CR Antibody Preparations Obtained from Immune Goat Serum to HK/68 HA Antigen*

Virus strains	Antibody preparations tested [a]							
	SS_{HK}		CR_{HK}		SS_{HK}		CR_{HK}	
	I[b]	II[b]	I	II	I	II	I	II
HK/68	16[c]	32	0 8	3 2	16	32	3 2	12 8
Eng/72	1 6	3 2	0 1	0 4	<0 1	<0 1	1 6	6 4
PC/73	0 2	0 8	0 1	0 8	<0 1	<0 1	0 4	1 6
Vic/75	<0 1	<0 1	<0 1	0 2	<0 1	<0 1	<0 1	0 2

a) See legend to Table 2
b) I Virus antibody interaction time for 5 minutes at room temperature II Virus antibody interaction time for 11 hours at 4°C
c) All entries are HI titres × 10^{3}

near this receptor region may be inconsistent with infecuvity Therefore the location of the more stable antigenic determinants namely the CR sites close to the distal part of the HA molecule might favour conservation of biological function In order to investigate whether CR antibodies are able to select antigenic mutants studies are now in progress to study antigenic drift *in vitro* and *in vivo* under selective immunological pressure by SS and CR antibodies

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subtype comprise CR-determinants which are antigenically common for a range of H3N2 viruses and thus relatively stable, and which may remain unchanged during antigenic 'drift'. In addition, there are other determinants which are more variable (*i e* the SS-determinants) and may change completely during antigenic 'drift' It is emphasized in this paper that the terms 'cross-reactive' and 'strain-specific' are operational and relative concepts and should therefore be used with reference to the actual cross-absorption experiments performed Thus, based on the experimental design of our assays, it appeared that the SS''_{HK}- and CR'_{HK}-antibodies were narrowly strain-specific and broadly cross-reactive, respectively On the other hand, both the SS'_{HK}- and CR''_{HK}-antibodies seemed to exhibit both cross-reactivity and strain-specificity when assayed in the various tests (SRD, IDD, HI) The closer, chronologically and antigenically, the absorbing virus was to the HK/68 variant, the more specific were the residual antibodies in the supernatant (SS'_{HK} versus SS''_{HK}) The more distantly related the absorbing virus was to the HK/68 strain, the more broadly reactive were the CR-antibodies (CR''_{HK} versus CR'_{HK})

The properties of the SS- and CR-antibodies, as shown in *HI tests (Table 3)*, suggested indirectly that the CR-antibodies were of low avidity, as previously shown by *Laver et al* (5), since the lack of reactivity vould be overcome by letting the virus and antibody interact for a long period of time (11 hours at 4°C) before adding the erythrocytes This increase in HI titres with time of reaction was demonstrated most clearly for the CR antibodies in our studies In terms of specific serological activity (*HI titre or SRD zone annulus area/µg* IgG protein), it was found that the SS-antibodies were *the most efficient type of antibody in both HI* and SRD tests (Table 4) This was also found to be the case in neutralization experiments *in ovo* and *in vivo* (3)

In SRD competition experiments, it is likely that the antibodies against determinants located in the more distal region of the HA spike, if added first, will interfere sterically with the binding of antibodies to determinants located somewhat nearer the proximal end of the molecule added subsequently,
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these
impose the same degree of sterical *hindrance*, the extent to which SRD zones produced by the anti-HA antibodies are *additive might shed some* light on the possible physical arrangement of the corresponding *antigenic determinants* on the HA subunit During these investigations it was found *that the CR-antibodies*, when applied to antigen first, may completely exclude the binding of the SS-*antibodies However, when the SS*-antibodies were added first, the interference with attachment of CR-antibod*ies was only partial (Table 5) This* observation supports the conclusions of *Laver et al* (5) that the SS- *and CR-determinants are not located on* different HA-spikes It has also been observed that anti-NA *and anti-HA antibody zones* were not additive in SRD tests (13), thus indicating that antibodies to determinants *on different surface* antigen molecules of influenza virus do not interfere mutually in these assays

Electron microscopic visualization of the sites of attachment of SS- and CR-antibodies on the influenza HA spike by *Wrigley et al* (20) has revealed that both types of antibody had become bound to sites located in the sub-tip region of the HA subunit and that this was indistinguishable for both types of antibody Our findings could be interpreted as suggesting that the CR-determinants are located closer to the distal end of the HA subunit than the SS-determinants This could explain the interference observed between the two populations of antibody when tested sequentially in SRD immunoplates A striking observation in our studies was *that when the SS'_{HK}- and CR''_{HK}-antibodies (shown* to be of intermidiate specificity) were tested for mutual additiveness, they seemed to interfere reciprocally to a similar degree, irrespective of which antibody was added first One possible explanation for this is that the SS'_{HK}- and CR''_{HK}-antibodies could be directed against overlapping *antigenic regions Others have also found* that there is a close spatial relationship between 'common' and *'specific' determinants* (9)

However, an alternative explanation for the *results* of our competition experiments is possible It was found consistently in SRD tests that more CR-antibody molecules were bound per virus particle than SS-antibodies (Table 4) since a given amount of SS'_{HK}-antibody gave larger zones than the corresponding amount of CR_{HK}-antibody Consequently, the patterns of mutual additiveness observed might therefore reflect a relatively higher density of *CR-antibodies bound per HA molecule than the SS*-antibodies, *thus suggesting that there are more CR*-than SS antibody-combining sites per HA molecule

It may be that the SS and CR-determinants represent regions on the HA molecule which undergo amino acid substitutions by mutation at a different rate or, more likely, show variable degrees of structural tolerance towards such substitutions Assuming that the HA receptor for the host cell resides at the distal tip of the HA molecule, rearrangements of the tertiary structure close to or

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GROUP L STREPTOCOCCI AS THE CAUSE OF BACTERAEMIA AND ENDOCARDITIS

A Case Report

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Bevanger L. & Stamnes T I Group L streptococci as the cause of bacteraemia and endocarditis A case report Acta path microbiol scand Sect B 87 301–302 1979

A beta haemolytic streptococcus was isolated from multiple blood cultures in a 56 year-old woman suffering from thrombophlebitis in a leg and from a 42 year-old man with endocarditis Both strains were identified as group L streptococci by precipitation testing Initially they were identified as group A streptococci on the basis of cultural characteristics bacitracin sensitivity and fluorescent *staining with* an anti group A conjugate The two cases are an addition to the few existing reports on proved infection in humans by group L streptococci

Key words Streptococcus Group L, bacteraemia endocarditis

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Group L streptococci have mostly been associated with infections in animals and infections in man have been reported only rarely Isolation of group L streptococci from two patients with bacteraemia one of them suffering from endocarditis is described here

CASE REPORT

Patient No 1 In 1977 a woman *born in 1921* was admitted to the Medical Department Regional Hospital Trondheim Norway In 1960 she underwent sugical treatment for cancer vulvae with removal of the inguinal lymph nodes Since then *she had* suffered from repeated episodes of thrombophlebitis in the left leg She h[illegible] [illegible] [illegible] oedema of the left leg but no other pathological findings Temperature was *38 3°C pulse rate 80* per minut blood pressure 170/100 white blood cell count $10\ 7 \times 10^9$ cells per l and erythrocyte *sedimentation rate 47* mm per h A beta haemolytic streptococcus was isolated from all of six blood cultures taken during the first 24 h She was treated with penicillin intravenously *and recovered* and has since been in good health Gynaecological examination and phlebography have revealed no abnormalities

Patient No 2 In 1978 a man *born in 1936* was admitted to the Medical Department Innherred Hospital Levanger Norway In March 1978 he had an acute febrile illness with sore throat and muscular tenderness and was treated with penicillin *He did not* recover completely and suffered from recurrent febrile episodes A systolic cardiac murmur was recorded some weeks later Physical examination revealed a holosystolic cardiac murmur *grade 3/4* Temperature was 38 1° C pulse rate 76 per min with premature beats blood pressure 155/85 white blood cell count 12×10^9 cells per l and *erythrocyte sedimentation* rate 34 mm per h Chest X ray showed moderate cardiomegaly and the diagnosis mitral insufficiency was recorded All four blood cultures taken shortly *after admission* showed growth of a beta haemolytic streptococcus The patient was treated with penicillin for six weeks and has since been in good health

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FIBRINOGEN BINDING STRUCTURES IN β-HEMOLYTIC STREPTOCOCCI GROUP A, C, AND G

Comparisons with Receptors for IgG and Aggregated β_2-microglobulin

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Binding of radiolabelled fibrinogen was measured to 197 strains of 16 different bacterial species All streptococcal strains belonging to groups A C and G isolated from human sources were strongly positive *S aureus* strains showed low binding values Occasional group B streptococci were positive Reactive strains were also noted among group C streptococci of animal origin *Streptococcus zooep demicus* and *Str equi* and bovine β hemolytic group G streptococci Bovine α hemolytic group G strains as well as the remaining seven species of human origin were all negative Inhibition experiments and correlation studies indicated that the streptococcal receptor for fibrinogen was different from immunoglobulin Fc binding reactivity Comparisons with the newly discovered β_2-microglobulin binding factor showed that trypsin concentrations which destroyed this receptor left the fibrinogen receptor intact Although the two receptors correlate in strain population studies and show competition for binding the difference in trypsin sensitivity indicates that they represent two different structural entities Both receptors might serve as basic markers for M protein like surface components of Gram positive cocci

Key words Streptococci fibrinogen IgG β_2 microglobulin

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Surface structures on several species of Gram positive cocci posses unique receptors specific for some mammalian proteins Protein A of *S aureus* can bind the Fc part of certain immunoglobulin classes and subclasses (5 6 7 17) A similar Ig binding receptor has also been found in streptococci groups A C and G (12) Fibronectin can bind to S [illegible] group A C and G streptococci as well as with other bacteria through Fab structures (4) Albumin has recently been shown to bind to a group G streptococcus (16) Among these newly discovered types of bacterial mammalian protein interactions the well known binding of fibrinogen to the clumping factor of *S aureus* should be included This interaction was discovered already in 1908 (22) A similar binding of fibrinogen to group A streptococ[illegible] to group A streptococci was shown to be M protein (8 9)

The presence of several different receptors for human proteins on group A C and G streptococci makes a localization of some of them to the same surface protein(s) rather likely Indeed the inhibition of binding of aggregated β_2-microglobulin by added fibrinogen suggests that the receptors for these two mammalian constituents may be identical or closely located on the same protein (13) The

Bacteriological Findings

The bacterial isolates from these two patients were identified as Lancefield group L streptococci by ring test precipitation and agar gel diffusion testing Hot formamide extracts from the bacteria and commercial group antisera (Wellcome) were used (4) The two strains could not be differentiated culturally or biochemically On blood agar colonies produced beta haemolysis similar to that of group A streptococci In fluid media moderately long chains of Gram positive cocci were observed When tested by Bacitracin differentiation discs 0 2 IU (Bio disk) a zone of inhibition of 10 mm was recorded The bacteria demonstrated a distinct fluorescent staining with an anti group A conjugate (Difco) The bacteria hydrolysed arginine Acid was produced from glucose maltose lactose sucrose and trehalose but not from arabinose mannitol sorbitol raffinose or salicin The CAMP test and tests for hydrolysis of aesculin or hippurate were negative The bacteria did not resist heat treatment at 60°C for 30 min and showed no growth at 45°C or on 40 per cent bile agar

DISCUSSION

Group L streptococci have been associated mainly with animal infections giving rise to bacterial endocarditis and pneumonia in pigs (7, 8) and genito urinary tract infections in dogs (6 8) Reports of human infections by these bacteria are few They include a patient with acute parotitis (2) a butcher with an infected finger (2) a patient with tonsillitis and bacteraemia (2) and three patients with subacute bacterial endocarditis (1 3) Bacteraemia by the group L streptococcus was verified in the two patients in this report one of whom was suffering from endocarditis

Patient No 1 had an underlying condition predisposing for recurrent thrombophlebitis She was often in contact with dogs which may have been the source of the streptococcus The thrombophlebitic leg is a possible portal of entry of the streptococcus though no skin lesion was observed Carditis was not diagnosed in this patient

Patient No 2 presented a classical history of subacute bacterial endocarditis and mitral insufficiency possibly induced or aggravated by the infection Contact with animals was denied by the patient The infection may have been endogenous since group L streptococci have been isolated from the throats of healthy humans (8)

The two group L strains isolated were initially identified as group A streptococci This was based on colony morphology and beta haemolysis Bacitracin sensitivity (though moderate) and fluorescent staining by an anti group A conjugate Antigenic determinants common to group A and group L isolates have been described (5) This may explain the cross reactivity shown by the anti group A conjugate In the precipitation tests no cross reaction between the anti group A and anti group L sera was observed Clearly, erroneous classification of group L strains especially as group A streptococci is a pitfall This may partly explain the rarity of proved human infections by group L streptococci

CONCLUSION

The cases reported emphasize the importance of recognizing the existence of more »uncommon« streptococcal groups in human infections and indicate the preferential use of precipitation testing in the identification of such streptococci

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[illegible] A bacterial surface protein with capacity to bind aggregated β_2-microglobulin has been detected in group A C and G streptococci (14) [illegible] group A [illegible] bacteria [illegible] recently [illegible] shown to bind to a group G streptococcus (16) Among these newly discovered types of bacterial mammalian protein interactions the well known binding of fibrinogen to the clumping factor of *S aureus* should be included This interaction was discovered already in 1908 (22) A similar binding of fibrinogen to group A streptococci was described in 1934 (30) and later also to group C and G streptococci (2) The bacterial structure responsible for fibrinogen binding to group A streptococci was shown to be M protein (8 9)

The presence of several different receptors for human proteins on group A C and G streptococci makes a localization of some of them to the same surface protein(s) rather likely Indeed the inhibition of binding of aggregated β_2 microglobulin by added fibrinogen suggests that the receptors for these two mammalian constituents may be identical or closely located on the same protein (13) The

present studies were performed in order to investigate the occurrence of fibrinogen receptors among various bacterial strains using sensitive techniques and to compare these receptors with binding structures for IgG and aggregated β_2 microglobulin Our results revealed extensive fibrinogen binding to group A C and G streptococci in a higher frequency than reported previously by others (8) The fibrinogen receptor on the surface of the bacteria was different from the other two receptors studied although a close localization to β_2 microglobulin binding sites seemed probable

MATERIALS AND METHODS

Bacterial strains A total of 197 bacterial strains from 13 Gram positive and 3 Gram negative species were included in the studies One hundred and sixtyseven strains of human origin were comprised of *Staphylococcus aureus* 18 strains *Staphylococcus saprophyticus* 10 *Staphylococcus epidermidis* 11 group A streptococci 20 group B streptococci 19 group C streptococci 20 group G streptococci 20 *Streptococcus faecalis* 10 *Streptococcus pneumoniae* 8 *Escherichia coli* 10 *Klebsiella pneumoniae* 10 *Pseudomonas aeruginosa* 11 All human strains were obtained consecutively from clinical specimens received at the Clinical Microbiology Laboratory University Hospital Lund Sweden *Streptococcus zooepidemicus* 8 strains *Streptococcus equi* 2 α hemolytic bovine group G strains 10 and β hemolytic bovine group G strains 10 were kindly supplied by Dr *O Holmberg* State Veterinary Laboratory Stockholm Dr *Martin Wierup* Veterinary School Uppsala Sweden and Dr *Roar Gudding* Veterinar institutet Oslo Norway Strains were subcultured on blood agar plates and then grown in tryptone broth or Todd Hewitt broth for binding studies

Protein preparations Human fibrinogen (AB Kabi Stockholm Sweden *Lot no 50293) was labelled with* 125 iodine using the electrolytic method of *Rosa et al* (27) as described by *Harboe & Folling* (6) The amount of radioactivity taken up by fibrinogen was approximately 1 8 kBq per μg protein The purity of the labelled preparations was also analysed Radiolabelled fibrinogen was added to whole human serum and to unlabelled fibrinogen and aliquots were tested in immunoelectrophoresis against rabbit anti human serum proteins and rabbit anti fibrinogen (code no 100 SF and 10 080 Dako A/S Copenhagen Denmark) followed by autoradiography The results confirmed *a localization of the*

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in phosphate
azide (PBSA)
for inhibition studies or radiolab[illegible] ith 125 iodine using the electrolytic method (27) for binding studies

Human β_2 microglobulin purified according to *Berggård & Bearn* (1) was kindly provided by Dr *Lars Björck* Lund Sweden Radiolabelling and glutaraldehyde aggregation followed methods described previously (14)

Binding assays Overnight broth cultures of bacterial strains were washed and suspended to 10^9 organisms per ml of phosphate buffered saline containing 0 1 % human serum albumin (AB Kabi Sweden) and 0 05 % Tween 20 (PSAT) Concentrations of bacteria were determined by measuring O D at 620 nm in a Beckman Colorimeter CP I Standard curves were produced from O D measurements of Petroff Hausser chamber counted standard suspensions of non aggregated bacteria For binding assays fibrinogen in amounts as indicated was mixed with 200 μl of bacterial suspension and incubated at room temperature for 60 minutes A 2 ml aliquot of PSAT buffer was then added and the bacteria sedimented by centrifugation at 2000 × g for 20 minutes The radioactivity of the pellet was measured in a gamma counter (LKB Wallac 1270 Rackgamma) and the binding expressed as per cent radioactivity remaining in the pellet Tests were performed in duplicates In preliminary experiments no further washings of the bacteria were found necessary in order to discriminate between positive binding and background uptake under the experimental conditions used Binding of human IgG or aggregated β_2 microglobulin was studied using similar assay systems as described previously (12 14 15 24)

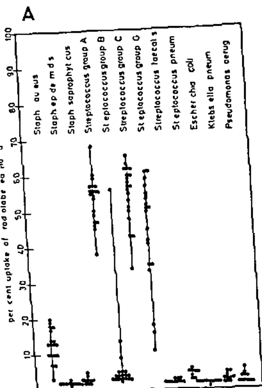
In inhibition tests varying amounts of unlabelled fibrinogen and human IgG was mixed with 1 μg of labelled fibrinogen The bacterial suspension was then added and the mixture incubated and processed as described for the binding assay

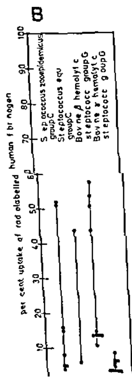
Trypsin digestion Varying amounts of trypsin (catalog no T 8253 Sigma Chemical Co St Louis Mo) was added to 1 ml suspensions of 10^9 bacteria in 0 25 M phosphate pH 7 5 and incubated for 20 minutes at 37°C The bacteria were then washed free of trypsin and tested for binding of fibrinogen Uptake of aggregated β_2 microglobulin was included as a control (14)

RESULTS

Binding of Human Fibrinogen to Bacterial Strains

The binding of radiolabelled fibrinogen to 167 bacterial strains of human origin was measured in a standardized assay using 5 μg amounts of labelled protein Fibrinogen was taken up by all group A C and G streptococcal strains studied (Fig 1A) The ranges of per cent uptake by the strains in the three groups were quite similar indicating a similar degree of fibrinogen binding in C and G strains as compared to that in group A strains *Staphylococcus aureus* strains also showed positive binding of fibrinogen in our assay system as expected because of the presence of clumping factor in this bacterial species (Fig 1A) The level of binding was much





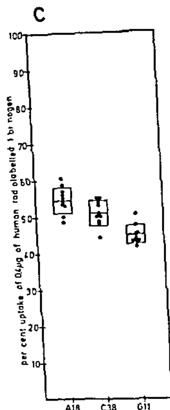
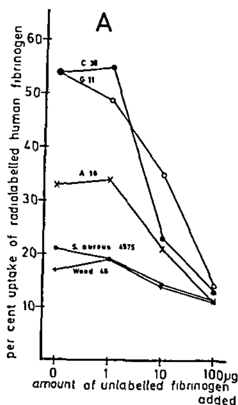
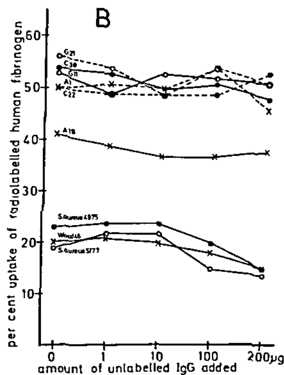


Fig 1 Binding of radiolabelled human fibrinogen (5 μg) to 197 strains of various bacterial species Binding is expressed as per cent uptake of added fibrinogen to the bacterial pellet (2×10^8 organisms) A Fibrinogen binding to 167 strains of twelve bacterial species of human origin 9 Gram positive and 3 Gram negative B Fibrinogen binding to 30 strains of animal origin Biochemical tests separate α and β hemolytic group G streptococci into separate species C Variations in fibrinogen binding studied by repeated culturing and testing of three different streptococcal strains with mean values and SD indicated

lower however than that recorded for streptococcal strains Among the remaining 8 bacterial species of human origin only occasional group B streptococci showed definite binding of fibrinogen

Among streptococci isolated from animal sources a significant binding of radiolabelled fibrinogen was noted in 4 of 8 strains of *Str zooepidemicus* 1 of 2 *Str equi* and in all ten bovine β hemolytic group G streptococci (Fig 1B) There was no uptake to α hemolytic group G strains There was an apparent two-group distribution among positive strains with one high level group binding 40–60 per cent and one low level group binding 10–20 per cent A tendency to a similar distribution can be seen among human group G strains (Fig 1A)





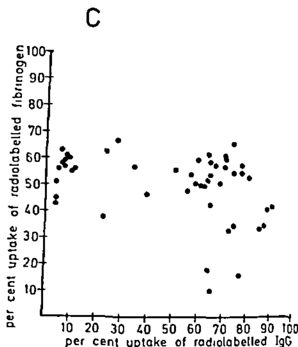


Fig 2A Inhibition of radiolabelled fibrinogen binding to *S aureus* and group A C and G streptococci (strains as indicated) by added unlabelled human fibrinogen B Inhibition experiments for studying the effect of added human IgG on the binding of radiolabelled human fibrinogen to *S aureus* (3 strains) and streptococci belonging to Lancefield s groups A, C and G (6 strains) C Levels of binding of human fibrinogen and normal human IgG was measured in 48 individual streptococcal strains group A, C and G The figures shows a complete lack of correlation between the two parameters

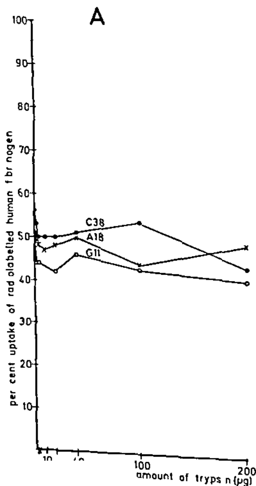
In order to determine the variability in the assay system, tests on repeated cultures were performed on three strains Fig 1C shows actual figures, mean values, and standard deviations, in these tests Only a slight variation is seen on repeated tests of these strongly fibrinogen-binding strains

Binding parameters The influence of incubation time on the binding of 1µg of radiolabelled fibrinogen to 2×10^8 reactive bacteria was studied Both strains of β-hemolytic streptococci *tested* (group A, strain no 18, group G, strain no 11) showed a maximal uptake of fibrinogen already after 2 minutes of incubation with no further increase up to 120 minutes The *Staphylococcus aureus* strain tested (no 4975) reached the maximum uptake only after 30 minutes of incubation or more The binding levels reached and the different effects of incubation time recorded indicate that the fibrinogen binding shown by streptococci is of higher avidity than that shown by the staphylococcal clumping factor

Serial dilutions of standard suspensions of three streptococcal strains with a suspension of a non-reactive *S epidermidis* strain (no D5244) were also tested for binding of radiolabelled fibrinogen At a dilution of 1/32 or more there was a marked drop in binding of 1 µg of fibrinogen indicating that the undiluted 200 µl sample provides a large excess of binding sites

Correlation to IgG receptors The possibility that iodination of fibrinogen induced or altered significantly the reactivity described was first investigated in inhibition experiments Varying amounts of unlabelled human fibrinogen was added in assays using three different streptococcal strains and two staphylococci The addition of fibrinogen suppressed the uptake of radiolabelled protein to all strains tested (Fig 2A) The iodination procedure therefore did not seem to affect the sequences responsible for binding or to induce such binding

Increasing amounts of human IgG were added in inhibition experiments using six streptococcal strains and three *S aureus* strains No significant inhibition of fibrinogen binding by IgG was seen for the streptococcal strains studied (Fig 2B) On the other hand the uptake of fibrinogen to *S aureus* was partially inhibited when a large excess of IgG was added (Fig 2B)



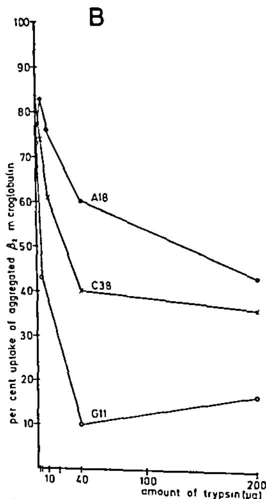


Fig 3 Trypsin digestion of three streptococcal strains group A C and G (strain numbers as indicated in the figure) followed by analysis of binding of human fibrinogen A) and aggregated β_2-microglobulin (B) shows a relative trypsin resistance of the fibrinogen receptor

The absence of a relationship between the fibrinogen receptor and IgG Fc binding structures on streptococci was further demonstrated in experiments comparing the quantitative binding of IgG (0 2 μg added to 2×10^8 bacterial cells) and of fibrinogen to individual strains As shown in Fig 2C there is no correlation between the binding capacity for the two human proteins

Further studies of the fibrinogen receptor The trypsin sensitivity of fibrinogen binding surface structures in streptococci was tested using amounts of enzyme ranging from 2 to 200 μg per ml of the standard bacterial suspension containing 10^9 orga

nisms Trypsin treated bacteria showed no change in fibrinogen binding as compared to untreated bacteria (Fig 3A) As a control the streptococcal strains were also tested for binding of aggregated β_2-microglobulin (14) In line with the marked trypsin sensitivity of the receptor for this protein there was a marked reduction of the uptake of β_2 microglobulin with increasing amounts of enzyme (Fig 3B) It is therefore clear that the relatively mild enzymatic treatment of the bacteria used does not affect fibrinogen binding structures known to reside in the M protein of group A streptococci From these results it is also clear that the fibrinogen receptor is different from β_2 microblobulin binding structures

The effect of heat treatment on the streptococcal fibrinogen receptor was also studied Suspensions containing $1\,2 \times 10^8$ organisms per ml of three streptococcal strains were treated for 30 minutes in water baths at different temperatures There was no effect of the heating procedure as tested up to 80°C on the binding of 0 4 µg fibrinogen to $2\,4 \times 10^7$ bacteria The same three strains (5×10^8 organisms per ml) were also formaldehyde treated at room temperature for 60 minutes and then tested for fibrinogen uptake With increasing concentrations of formaldehyde there was a slight reduction in the binding capacity

DISCUSSION

Fibrinogen binding in group A streptococci was originally described by *Tillett & Garner* (30) In later studies by *Duthie* on the staphylococcal clumping factor a similar binding was also seen in A C and G streptococci (2) Reports by *Kantor & Cole* (8) and later by *Kantor* (9) have demonstrated that fibrinogen binding in group A streptococci is mediated by M protein With their precipitation method only 69 of 131 strains were positive Using a more sensitive isotope technique we have demonstrated fibrinogen binding in all 60 group A C and G streptococcal strains tested Our isolates were obtained from routine specimens A slightly lower percentage was obtained when selected laboratory strains were analysed (13) The present experiments indicate that group C and G streptococci of human origin carry a fibrinogen receptor undistinguishable from the group A receptor as far as degree of binding inhibition pattern lack of relation to other known receptors trypsin resistance heat and formaldehyde resistance is concerned Group A C and G streptococci are in many ways similar and with

... they all seem to share

... have shown that

... t also fibrinogen preparations from other mammalian species were reactive (8) This has been confirmed earlier in gel diffusion experiments showing interactions in a reference gel precipitate (M1 – anti M1) by plasma samples from gorilla orang utan polar bear sun bear siberian tiger cheetah west coast harbour seal and antelope but not from corresponding serum samples (*G Kronvall B F Anthony U S Seal & R C Williams Jr* 1969 unpublished observations)

In addition to fibrinogen binding in *S aureus* strains and in group A C and G streptococci as known previously (2 8 9 30) we have also detected such binding activity among group B streptococci and in three species of streptococci from animal sources Three out of 20 group B streptococci were positive one of them with an uptake as high as 55 per cent Two of eight *Str zooepidemicus* strains one of two *Str equii* and all ten bovine group G β hemolytic streptococci were positive The last species resembles human A C and G streptococci in having an IgG binding receptor in 13 out of 16 strains tested (23) This receptor is clearly different from the type II and III receptors in group A and group C and G strains respectively (23 24 25) The relationships are emphasized however by the presence of fibrinogen binding structures in all of them indicating structural similarities between surface proteins of these four species

Reactive surface structures in group A C and G streptococci for three types of human proteins were compared in order to determine their possible identity or their simultaneous presence on the same surface protein molecules (12 14 30) Trypsin digestion experiments differentiated between fibrinogen binding and uptake of aggregated β microglobulin the latter being extremely sensitive Earlier experiments have shown inhibition of β_2 microglobulin binding by added fibrinogen (13) Receptors for these two proteins therefore seem to be located on different but adjacent parts of the same bacterial surface proteins in streptococci Comparisons of fibrinogen binding and IgG uptake showed another picture Inhibition experiments using an excess of human IgG were completely negative for the streptococcal strains tested Since difficulties might be expected in such experiments due to a rather low affinity of IgG for the corresponding receptors additional tests were also performed When binding of the two proteins to individual strains were compared an apparent lack of correlation was readily demonstrated *Therefore* the receptors for fibrinogen and for human IgG on group A C and G streptococci seem to be completely different from each others The rare occurrence of haptoglobin binding precludes the

possible identity of this receptor with any one of the other three

Fibrinogen binding structures in group A strains are known to be identical to M protein (9) Our results suggest that similar surface proteins with identical fibrinogen binding structures are present also in group C and G streptococcal strains It has been shown by others that M proteins of different M types share antigenic determinants (32, 33) M antigens have also been detected in group G streptococcal strains (21) It is therefore clear that conventional criteria for M protein based on antiphagocytosis long chain reaction and the definition of specific M antigens do not fulfill the need for a more basic definition suitable for use in chemical and immunological studies of bacterial surface proteins (3 29 31) Fibrinogen binding might represent a useful and easily detectable marker for M protein like surface structures The biological effects usually referred to as markers might be due to mutational variations in parts of a more well-conserved structural backbone The existence of more stable parts of the various types of M proteins in common with most A C and G streptococci provides a basis for a better definition of these surface proteins which is required in more general studies of the biochemical composition of cell wall associated structures

Several mammalian proteins are now known to interact specifically with surface structures of *S aureus* strains and group A C and G streptococci phenomenon which have been termed »short circuits to illustrate unexpected intrusions into common pathways of mammalian reactivities (13 18) Immunoglobulin G (5 12) IgD (4) IgE (7) albumin (16) fibrinogen (2 8 9 22 30) haptoglobin (26 31) fibronectin (19) are all capable of direct binding to bacterial structures Aggregated β_2-

yet clearly understood In analogy to mimicry of the host as described in schistosomiasis the coating of the bacteria with host material might facilitate an escape of normal recognition mechanisms (28) It is also possible however that short circuiting a

circuits and a detailed analysis of mechanisms involved might add new dimensions to our understanding of parasitic as well as saprophytic infestations

The assistance of Mr *Åke Christensson* for preparing the illustrations is kindly acknowledged

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Acta path microbiol scand Sect. B 87 311–315 1979

PLEURAL EFFUSION DISEASE IN RABBITS

Interferon in Body Fluids and Tissues after Experimental Infection

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Animal Department Statens Seruminstitut, Copenhagen and Institute of Medical Microbiology* Aarhus Universitet Aarhus Denmark

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The distribution of interferon in body fluids and tissues was studied in 18 rabbits infected experimentally with the agent of pleural effusion disease (PED) Circulating interferon of the classical type was demonstrable 12 h after inoculation and a maximum response was attained 2–3 days later Circulating interferon disappeared between 6 and 8 days after inoculation Interferon titres of serum were closely correlated with the early phase of febrile response and probably also with the initial growth phase of the PED agent The interferon titres of pleural fluid exceeded by far the titres of other body fluids and tissues No interferon could be demonstrated in brain liver and urine

Key words Interferon viral infection pleural effusion disease rabbit

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contaminant of rabbit testicular suspensions of *Treponema pallidum* in the Scandinavian countries (6 12)

Fennestad et al (3) demonstrated that the PED agent could readily be propagated in rabbits by serial passages at intervals of 2 to 30 days They considered the infection to be a new rabbit disease and presented evidence supporting the hypothesis that the PED agent originally was imported to Denmark in 1951 from U S A being carried in a rabbit as a passenger with the Nichols pathogenic *T pallidum* Later on the PED agent was probably brought to Sweden and Norway with the Nichols strain from Denmark This hypothesis implies that the PED agent occurs also in other countries.

There is very little information regarding the nature and properties of the PED agent itself *Gudjonsson et al* (8) reported that the PED agent will pass Millipore filters with a pore size of 0 01 μm and that the [illegible]

37 °C for 24 h and at minus 20 °C for at least five years without apparent decrease in infective titre (4)

Gudjonsson et al (7 8) also reported on the isolation of an agent from liver and testes of one infected rabbit This agent induced a cytopathic effect in primary rabbit kidney cell cultures and could be carried through at least five passages The isolate was proposed either to be related to *Herpes simplex cuniculi* or to be some other natural virus pathogen of rabbits Our own attempts to demonstrate the PED agent in two different primary rabbit cell cultures and in a few permanent cell cultures have failed hitherto (1)

The present experiments were carried out in order to study the production of interferon in rabbits infected experimentally with the PED agent

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The present experiments were carried out in order to study the production of interferon in rabbits infected experimentally with the PED agent

MATERIAL AND METHODS

PED agent The source and the passage of the Danish PED agent in rabbits have been described elsewhere (3) The inoculum for the present experiments was a pooled *serum originating from five rabbits* given a subcutaneous injection of approximately 10^4 rabbit infective doses 48 h previously The pooled serum was stored at minus 70 °C until use The term rabbit infective dose refers to a dose capable of producing a typical clinical response and/or preventing the appearance of a typical clinical response on challenge 30 days after inoculation The number of rabbit infective doses of the PED agent per ml was determined by making serial ten fold dilutions using only one rabbit per dilution

Experimental infection of rabbits Eighteen male albino rabbits (Ssc CPH) weighing 2 0 to 2 5 kg were used in two experiments They were provided with a pelleted diet and water *ad libitum* Each animal was inoculated subcutaneously on day zero with 1 ml of the above mentioned serum pool diluted 1 10 in PBS (pH 7 0) corresponding to approximately 10^4 rabbit infective doses

After inoculation the animals were observed for fever and other clinical signs of disease and animals that died were examined as described previously (3)

Samples to be examined for interferon were obtained in two differently designed experiments In a preliminary experiment (Exp 1) blood samples from six rabbits were collected and serum obtained before inoculation and at intervals of 12 h after inoculation

In the succeeding experiment (Exp 2) *serum samples* from 12 rabbits were obtained before inoculation 36 h after inoculation on post inoculation (p i) days 6 8 10 14 19 and 30 and at death on p i days 3 to 9 At the *post mortem* examination samples of brain anterior eye chamber fluid thymus lungs pleural fluid liver spleen kidneys urine and popliteal lymph nodes were collected From the two surviving animals samples of serum anterior eye chamber fluid and urine were obtained at sacrifice on p i day 30 All samples were stored at minus 70 °C until examined for interferon

Organ extracts A 10% suspension was made directly from frozen organs by grinding them with sand in PBS (pH 7 2) After centrifugation at 6000 rev/min for 30 min the supernatant was withdrawn and used for interferon assay

Preparation for interferon assay The serum and other body fluids organ extracts and tissue culture media were dialysed against Sørensen s buffer pH 2 and after 48 h at 4 °C dialysed back to pH 7 4 Some of the samples were centrifuged at 110 000 *g* for 3 h at 4 °C

Cell culture The culture used was a continuous rabbit kidney cell line (RK_{13}) originally received from Dr *Annelise Godtfredsen* Statens Seruminstitut Copenhagen The cells were grown in one litre Roux flasks using Eagle s MEM with 15% calf serum as growth medium and Eagle s MEM with 2% calf serum as maintenance

Interferon assay A micromethod was u... Microtest II (Falcon) wells were seeded with RK_{13}-cells – 30 000 cells per well in a volume of 0 1 ml After growth to confluence serial three fold dilutions of the serum organ suspensions or tissue culture media to be tested were made directly in the wells Twenty four h later the cells were challenged with vesicular stomatitis virus (5000 pfu in 0 1 ml/well) and cytopathic effect was read microscopically after incubation for 24 h The interferon titre represented the dilution that reduced the cytopathic effect by 50% All samples were tested in duplicate rows An interferon standard of known titre was included on each plate in order to correct for fluctuations in the sensitivity of the system

Control interferon was induced in 2–3 kg rabbits by double stranded polynucleotide Poly I Poly C (Miles Laboratories) The compound was dissolved in PBS (pH 7 3) at a concentration of 1000 U/ml Each rabbit was given 1 ml intravenously Blood was taken from an ear vein after 2 h The serum was dialysed against *Sørensen s buffer pH 2 and after 48 h at 4 °C* it was dialysed back to pH 7 4

Characteristics of viral inhibitor The viral inhibitor found in the dilutions of body fluids and organ extracts was stable at pH 2 for 48 h partly inactivated at 56 °C for 30 min and inactivated by trypsin No changes in the viral inhibitory effect was found after centrifugation at 110 000 *g* for 3 h at 4 °C This indicates type I interferon or classical interferon (16)

RESULTS

Fever and other clinical signs of PED were demonstrable on p i day 2 in all but one (dying on p i day 3) of the 18 rabbits

In the first experiment all six rabbits died on p i days 3 or 4 and in the second experiment 10 of the 12 rabbits died on p i days 3 to 9 *Post mortem*

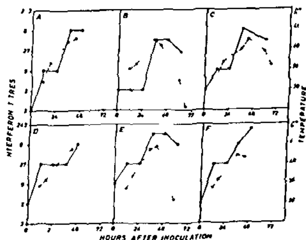


Fig 1 Early interferon titres of serum and body temperatures in six rabbits (Exp 1)
Interferon ●——●
Body temperature ○——○

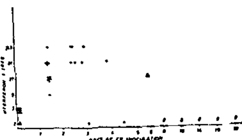


Fig 2 Interferon titres of serum from 12 rabbits followed until death or sacrifice (Exp 2)

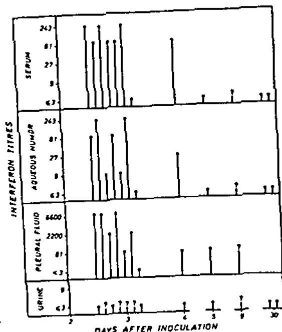


Fig 3 Interferon titres of serum aqueous humor pleural fluid and urine at death or sacrifice of 12 rabbits (Exp 2) Each disconnected vertical line represents one rabbit.

examinations revealed gross changes typical of PED in all the dead animals The two surviving rabbits recovered from their clinical disease in the third week and were apparently normal when sacrificed on p i day 30 Nevertheless, titration of serum samples from these two rabbits revealed a concentration of approximately 10^3 rabbit infective doses of the PED agent per ml of serum

Interferon in body fluids Fig 1 illustrates the interferon titres of serum and the body temperatures of six rabbits from time of inoculation until death between 48 and 72 h p i It appears that interferon was present already 12 h p i and that peak titres probably were attained before death in three of the six animals The figure also shows that rising interferon titres coincided with the onset of the febrile response

Fig 2 shows the interferon titres of serum from 12 rabbits observed for a longer period Peak titres similar to those in the first experiment were observed between p i days 2 and 3 Interferon was still demonstrable on p i day 6 in three surviving rabbits, but not on p i day 8 or later in the two animals which survived the infection Unfortunately serum from day 9 from one animal (death on day 9) was unsuitable for detection of interferon

Fig 3 shows the relationship between interferon

TABLE 1 *Distribution of Interferon in Tissues at Death of 10 Rabbits (Exp 2)*

Rabbit	Examined at p i h	Interferon titres of tissues[a]						
		Brain	Lung	Thymus	Spleen	Liver	Kidney	Popl lymph
Q	60–74	<30	270	90	270	<30	<30	270
J	"	<30	90	90	[b]	<30	<30	< 30
R		<30	270	270	810	<30	<30	90
K		<30	270	30	270	<30	<30	< 30
M		<30	270	30	90	<30	<30	< 30
I		<30	270	810	< 30	<30	90	90
O		<30	30	30	< 30	<30	<30	< 30
N	94–107	<30	< 30	< 30	[c]	<30	<30	< 30
G	"	no exam	< 30	< 30	< 30	<30	<30	no exam
H	192–195	<30	< 30	< 30	< 30	<30	<30	< 30

[a] Interferon content per gramme of tissue calculated from titres of 10% suspensions
[b] Toxic in dilution 1 90 and below no virus inhibiting activity in higher dilutions
[c] Toxic in dilution 1 30 no virus inhibiting activity in higher dilutions

titres of serum and other body fluids at time of death or sacrifice of the 12 rabbits As will be seen, the titre level in aqueous humor was similar or slightly lower than the titre level in the corresponding serum The highest titres of interferon were observed in the pleural fluid, which contained interferon in concentrations up to about 80 times higher than the corresponding serum No interferon could be detected in the urine samples examined in dilutions 1 3 or 1 9

Interferon in tissues Table 1 shows the results of titrations of interferon *in various tissues from 10* rabbits at the time of death, the sequence of the animals being the same as *shown in Fig 3 In* animals that died early in the infection, interferon *was demonstrated regularly in the lung*, thymus, spleen and popliteal lymph node, but not in the *liver or brain in dilution 1 30 In* only one of these animals interferon was also found in the kidneys At *p i h 94* or later, interferon was no longer demonstrable in dilution 1 30 in any of the tissues examined

PED agent in mice and tissue culture In order to evaluate if the PED agent could induce interferon in mice, each of 42 2½-month-old male mice (Ssc AH) was injected intravenously with approximately 10^3 rabbit-infective doses of the PED agent No interferon could be demonstrated in serum dilution 1 9 from these mice, when they were sacrificed in the period from 2 to 168 h after injection The RK_{13} cells were inoculated with various dilutions of the PED agent and the medium was harvested 6–96 h after inoculation No virus inhibiting activity was found in any of the tissue culture media

DISCUSSION

The virus inhibiting activity found in body fluids and organs from the rabbits in the course of experimental PED infection was characterized as classical interferon (16, 17) Such interferon is generally considered to be the result of multiplication of a virus This supports the proposition that PED infection has a viral aetiology (13) By inoculation of tissue cultures with the PED agent no virus inhibitory activity was induced, probably because of the absence of multiplication of the agent in tissue culture The lack of induction of interferon in mice after inoculation of the PED agent may express a low interferon inducing capacity of the agent, but may also indicate an insufficient dose of the PED agent (10) Previous *experiments with* titration of rabbit serum from various stages of experimental pleural effusion disease *have indicated* a gradual increase in the number of rabbit infective doses from approximately $10^{2.5}$ to 10^6 per ml serum in the period from 12 to 72 h p i (4) This suggests a close relationship between interferon production and the early growth phase of the PED agent Evidently, *the interferon production did not run* parallel with the virus titres at a later stage of *infection, since interferon in serum apparently* disappeared between p i days 6 to 8 while the two *surviving rabbits still had a rabbit infective titre of* approximately 10^3 per ml serum on p i day 30

When production of interferon has been followed in experimental virus infections, the content was most often correlated with the intensity of infection of organs (9), although there seems to be a tendency for organs with high content of lymphoid cells to have a higher interferon production (9) From this point of view, the main target organs for the pleural effusion disease agent would be the lungs and pleura, and to a lesser extent the eyes thymus spleen, lymph nodes and perhaps the lymphoid cells of the blood

In PED infection the most prominent gross lesion is the accumulation of fluid in the pleural cavities This fluid is rich in protein and almost cell free, and is considered to be the result of an increased permeability of the pulmonary or parietal pleural capillaries, or both (2) The comparatively high titres of interferon in pleural fluid might therefore be taken as an index of the extent of multiplication of the PED agent in the lungs and/or pleura Another explanation for the high titres might be an escape of interferon from the general circulation to the pleural cavities, perhaps combined with a less rapid clearance of interferon from the pleural fluid The latter explanation seems less likely In the anterior eye chamber fluid the protein concentration is considerably increased thus indicating a faulty blood-aqueous barrier (5) Nevertheless the interferon titres of the anterior eye chamber fluid never exceeded the level found in serum

The lack of demonstrable interferon in the brain and liver may indicate a low or a *completely lacking* multiplication of the PED agent in these organs, although *it cannot be excluded that interferon* produced in the liver was decomposed by enzymes prevalent in that organ *(15) The finding of* interferon in the kidney of only one of the rabbits shows *that this organ is seldom the site for* multiplication of the PED agent Interferon was *never demonstrated in urine Admittedly*, urine from some of the rabbits was toxic in dilution 1 3, *but it was always negative in a three fold higher* dilution It has been found previously that renal excretion is not the main pathway by which

interferons are eliminated from the body and only if very high titres are found in the serum will it be possible to demonstrate interferons in the urine (11)

The quite close correlation between the occurrence of interferon in serum and the body temperature during the initial stage of pleural effusion disease has to the best of our knowledge never been observed in naturally-occurring or experimentally produced animal infections by replicating agents In man however some correlation has been demonstrated (14)

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Acta path microbiol scand Sect. B 87 317–319 1979

BRIEF REPORT

EXPERIMENTS TO ILLUSTRATE THE EFFECT OF CHLORPROMAZINE ON THE PERMEABILITY OF THE BACTERIAL CELL WALL

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Kristiansen Jette E H Experiments to illustrate the effect of chlorpromazine on the permeability of the bacterial cell wall Acta path microbiol scand Sect B 87 317–319 1979

The present investigation has been made to illustrate whether the cell walls of micro-organisms are affected by membrane stabilizers *In vitro* experiments were carried out with *S aureus* under the influence of chlorpromazine (CPZ) De pigmentation and a bacteriostatic and bactericidal effect of CPZ on the micro-organisms were seen It has been shown that concentrations of CPZ near the bacteriostatic value in combination with bacterial haemolysins alters erythrocyte membranes (horse and rabbit) in such a way that they become resistant to haemolysis It has been shown that CPZ in bacteriostatic concentration probably changes the transport of potassium through the bacterial membrane in the same manner as described for mammalian muscle tissue

Key words *S aureus* alfa and delta haemolysins membrane stabilizers chlorpromazine

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Received 12 vii 79 Accepted 7 viii 79

The group of drugs called membrane stabilizers (11) comprises different chemical compounds of which a [illegible] mammalian muscle tissue and erythrocytes has been shown by many investigators (2 7 10 12) to be a reduction of the permeability of cell membranes for water molecules and ions (sodium and potassium) Glucose transportation through the membrane is also altered (2 3)

Since membrane stabilizers and antimicrobial agents are often used simultaneously it was considered of interest to study the effect of these compounds on bacterial cell walls Only results obtained with chlorpromazine and a few selected strains of *S aureus* will be reported here

Materials and Methods

Bacterial strains Three strains of *Staphylococcus aureus* isolated from patients with bacteraemia were studied The phage types were 52/80/81 (strain U) 89u (strain V) 29u 100×RTD (strain X) the Blair & Williams method were used (1) Strain V produces alpha haemolysin strain X delta haemolysin and strain U both haemolysins (8) For strain U only results obtained in growth inhibition experiments are included

Membrane stabilizer Discs (Schleicher and Schull No 2668-6mm) containing 800 μ chlorpromazine (CPZ) (D A K) were prepared

Growth inhibition experiments

a) Solid medium A CPZ disc was placed on brothpeptone agar medium containing 5% horse blood and was removed again after incubation for 20h at 4 °C Using a bent glass rod the medium was inoculated evenly with 0 1ml of a bacterial suspension containing 10^6 colony forming units (CFU) per ml and incubated at 35 °C for 20h

b) Fluid medium An 18h broth culture was diluted to a concentration of 10^7 CFU/ml aliquots of 10 ml were distributed into six tubes to each of which CPZ was added to a final concentration of 0 50 75 100 150 200 μg/ml respectively The tubes were then incubated in a waterbath at 37 °C Determinations of CFU for each sample were made after incubation for 0 2 4 and 24h The results are given as $\log_{10}$ to the number of CFU/ml

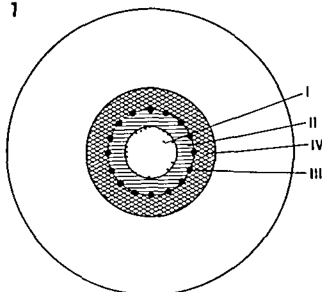


Fig 1 Schematical drawing of the various zones
I Complete haemolysis and no bacterial growth
II No visible growth
III Non pigmented colonies
IV Normally pigmented colonies
II + III + IV The medium has become opaque and the colour has changed to a darker red

Demonstration of the effect of CPZ on alpha and delta haemolysis

Broth peptone agar medium containing 5% rabbit blood or 5% horse blood was used According to *Elek* (6) the alpha haemolysin lyses rabbit erythrocytes but not horse erythrocytes the zone of haemolysis is broad and the delineation is diffuse Delta haemolysin forms a narrow and well defined zone of haemolysis on both types of medium The CPZ disc was placed asymmetrically between two streak inoculations of staphylococcal cultures to be tested and the plates were incubated for 24h at 35 °C (see Fig 3 and 4)

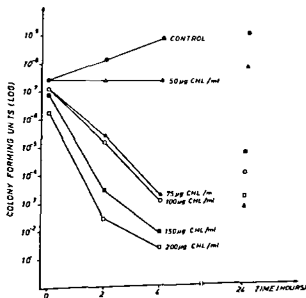


Fig 2 Effect of CPZ on the growth of *Staphylococcus aureus* strain (U)

Results

Effect of CPZ on bacterial growth and haemolysis using solid media The pattern characteristic for haemolysin producing strains is shown schematically in Fig 1 A zone (I) about 12 mm in diameter around the disc showed complete haemolysis and no bacterial growth Zone I was surrounded by a zone without visible growth (II) a ring of non pigmented colonies (III) and a zone of normally pigmented colonies (IV) As regards zones II III and IV the medium had become opaque and the colour had changed to a darker red This phenomenon did not occur on non inoculated media incubated with the CPZ disc alone The remaining area was covered by normal pigmented growth which produced normal haemolysis of the erythrocytes in the medium

Bactericidal effect of CPZ In order to obtain a quantitative measure of the bactericidal effect of CPZ growth inhibition experiments were performed as described under Materials and Methods The results obtained for strain U are shown in fig 2 At a concentration of 50 µg per ml CPZ was bacteriostatic and at higher concentrations a bactericidal effect was demonstrated Similar results were obtained with other strains of *S aureus studied*

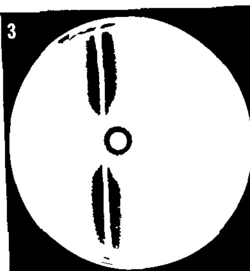
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Discussion

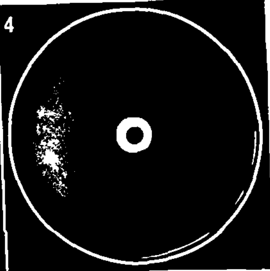
This is a preliminary report on the effect of CPZ on the permeability of bacterial cell walls or membranes It has been shown that CPZ in concentrations near the bacteriostatic value in combination with bacterial haemolysins alters erythrocyte membranes (horse and rabbit) in such a way that they become resistant to haemolysis Simultaneously the release of haemolysins from the bacteria and/or the production may be diminished It has also been shown that CPZ in bacteriostatic concentration probably changes the transport of potassium through the bacterial membrane in the same manner as described for mammalian muscle tissue (to be published later) The bactericidal effects of the phenothiazin derivator *CPZ* have been described previously (4 9)

De pigmentation of *S aureus* occurs regularly under suboptimal growth conditions (5)

A change in the permeability of the bacterial cell wall or membranes would be expected to alter the susceptibility to anti microbial agents so that either a synergistic (13) or an antagonistic effect would occur The combined



Strains (V) and (X)



Strains (X) and (V)

Fig 3 The alpha hemolysin producing strain (V) and the delta hemolysin producing strain (X) on 5% rabbit blood agar under the influence of CPZ

Fig 4 The delta hemolysin producing strain (X) and the alpha hemolysin producing strain (V) on 5% horse blood agar under the influence of CPZ

use of psycopharmaca and antibiotics is common Therefore a further investigation of this interrelationship is called for

I wish to thank Dr *J Bang* Dr *I Lind* and Dr *K Rosendal* for helpful suggestions

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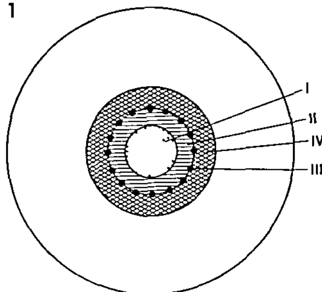


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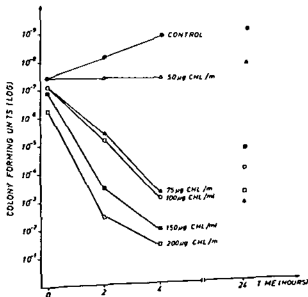


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SURFACE PROPERTIES OF *STAPHYLOCOCCUS SAPROPHYTICUS* AND *STAPHYLOCOCCUS EPIDERMIDIS* AS STUDIED BY ADHERENCE TESTS AND TWO-POLYMER, AQUEOUS PHASE SYSTEMS

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Department of Urology[1] University Hospital Lund and Department of Medical Microbiology[2] and Department of Microbiology[3] University of Lund Lund Sweden

Colleen S Hovelius B Wieslander Å & Mårdh P A Surface properties of *Staphylococcus saprophyticus* and *Staphylococcus epidermidis* as studied by adherence tests and two-polymer aqueous phase systems Acta path microbiol scand Sect B 87 321–328 1979

S saprophyticus is an established pathogen in man devoided of characteristics associated with pathogenicity in *Staphylococcus aureus* The ability of this species to attach to cells from regions viz the urinary tract where it acts as an invador and to cells from areas where it is known as a commensal was compared to its behaviour in this respect with another staphylococcal species viz *S epidermidis* *S saprophyticus* showed a preferential adherence to human exfoliated urogenital cells when compared with its ability to attach to skin and buccal cells from man and also when compared with porcine cells from these regions The profound ability to adhere to human exfoliated urogenital epithelial cells by far exceeded that of *S epidermidis* while no such species difference was found when testing porcine cells (*S saprophyticus* is unknown as a urogenital tract pathogen in pigs) When studied in a two polymer aqueous phase system *S saprophyticus* and *S epidermidis* were found to have a negative surface charge at pH 7 2 but the former carried a considerably higher surface charge density Both staphylococcal species exhibited a poor hydrophobic interaction liability These physico chemical surface characteristics are briefly discussed with regard to the differential bacteria-cell interactions of these species

Key words *Staphylococcus saprophyticus* physico-chemical surfaces properties bacteria-cell interaction

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Accepted as submitted 9 v 79

Staphylococcus saprophyticus is a coagulase negative novobiocin resistant staphylococcus which is a common cause of acute often hemorrhagic cystitis particularly in young women (15) Infection with this organism may also engage the upper urinary tract (9 14)

In an earlier study (18) we found *S saprophyticus* to adhere in greater number per urothelial cells than bacteria of several other species known to cause urogenital tract infections except for gonococci

S saprophyticus lacks characteristics considered as possible pathogenicity factors in *Staphylococcus aureus* (21) In contrast to other staphylococcal species most strains of *S saprophyticus* cause direct hemagglutination of sheep erythrocytes (8)

Partition in a two-polymer aqueous phase system have proved to be useful for the separation of cells and subcellar particles and also to characte-

absolute comparison between different cells or particles with regard to the surface property studied (1)

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Partition in a two-polymer aqueous phase system have proved to be useful for the separation of cells and subcellar particles and also to characterize the cell surface with regard to charge charge density and hydrophobic properties (1 2 28) The use of this system in a counter-current distribution further increases the resolution and allows an absolute comparison between different cells or particles with regard to the surface property studied (1)

In the present study, mechanisms which may explain the tropism and pathogenicity of *S saprophyticus* for the urinary tract in man are discussed The ability of this bacterium to attach to human and porcine epithelial cells of different origin including exfoliated urothelial cells was studied in *in vitro* experiments Urine sediments of women with acute urinary tract infection (UTI) caused by *S saprophyticus* were studied to elucidate cell attachment of the bacterium in the naturally diseased host Cell surface characteristics of *S saprophyticus* were studied by partition in two polymer, aqueous phase systems utilizing counter-current distribution The possible significance of these characteristics in the pathogenicity of *S saprophyticus* for the urinary tract is discussed For comparison similar experiments were performed with *Staphylococcus epidermidis*

MATERIAL AND METHODS

Adherence Tests

Bacteria One freshly isolated strain each of *S saprophyticus* and *S epidermidis* recovered from human voided urine specimens were tested The bacteria used were harvested from blood agar plates consisting of Blood agar base No 2 (Oxoid) containing 4 per cent defibrinated horse blood and identified as to species according to *Kloos & Schleifer* (13)

Cells Exfoliated cells from the urogenital tract were recovered from freshly voided urine specimens of two males and five females without history or signs of urogenital disease The mean age of the persons studied was 31 years (range 22–40 years) Buccal cells and cells from the periurethral area were obtained from the females by gentle scraping of the mucosal lining with an Ayre s spatula From all persons studied cells were also collected with a scalpel by scraping the skin between the shoulders The cells were suspended in Minimal essential medium (MEM) (Flow Laboratories Ltd) pH 7 2

Buccal and skin cells from a boar and sow were also tested The cells were collected as described above immediately after the animals had been killed in a slaughtery house Urothelial cells from the animals were recovered from urine collected by bladder puncture

Test procedure The cell suspensions were passed through a membrane filter with a pore size of 14 μm (Millipore Ltd) a pore size which allows bacteria debris and crystallized material to pass but will retain epithelial cells Immediately after the specimens had been filtered another syringe containing fresh MEM was fitted to the filter holder and the cells were washed This procedure was repeated twice The cell suspension was diluted in MEM to 10^5 cell/ml The bacteria were ... MEM

determined in a *Burker counting* ...

(2 1) of the bacteria and cell suspension was incubated at 37 °C for 30 minutes The adherence tests were otherwise performed as described in detail elsewhere (18) The number of bacteria that had adhered per cell was determined by counting the organisms under a light microscope (× 720) after staining with methylene blue

Hemagglutination Tests

Direct hemagglutination of the two strains of *S saprophyticus* used in the partition tests were performed as described elsewhere (8) using 10 per cent suspension of washed bacteria in phosphate buffered saline (PBS) pH 7 2 In the agglutination tests a one per cent suspension of sheep erythrocytes was employed

Partition Tests

Bacteria Two strains of *S saprophyticus* and three strains of *S epidermidis* were studied

Treatment of bacteria The organisms were incubated in 50 ml of Trypticase soy broth (Oxoid) for 4 / hours at 37 °C The cultures were then centrifuged at 32 000 × *g* for 10 minutes at 5 °C and the pellets suspended in 10 ml saline The optical density of the suspensions were measured at 540 nm (OD_{540}) The suspensions were then washed twice in 10 ml 0 32 M sucrose Finally the pellets were suspended in 3 75 ml of a mixture (8 7) of predetermined parts of top and bottom phases for counter current distribution (CCD) experiments (see below) The last three procedures were all performed at 4 °C

Phase systems The phase system was composed of 5 per cent (w/w) dextran T 500 mol wt 500 000 (Pharmacia Uppsala Sweden Lot No 4049) 4 per cent (w/w) polyethylene glycol (PEG) Carbowax mol wt ... York) 5 mmol

charge dependent 50 mmol kg Na_2SO_4 ...

To obtain a non charged (hydrophobic) phase system 100 mmol kg $^{-1}$ NaCl instead of sulphate was used After mixing the components the phases were allowed to equilibrate over night at 4 °C The top and the bottom phases were then separated and stored at 4 °C until use

Counter current distribution experiments The bacterial preparations were suspended in 3 75 ml of a top and bottom phase (8 7) mixture (load mixture)

An automatic thin layer counter current distribution apparatus with 120 cavities (numbered 0–119) was used (1) Cavities 0–1 and 40–41 received 1 48 ml each of one of the load mixtures as did when needed cavities 80–81 All the other cavities received 0 69 ml bottom phase and 0 79 ml top phase Thirty-eight transfers were completed at 4 °C using a settling time of 8 minutes and a shaking time of 20 seconds

After the counter-current run 1 0 ml distilled water was added to each cavity to convert the phase systems to homogeneous (one phase) suspensions before transferred to test tubes The OD_{540} of the one phase suspensions was then determined

TABLE 1 *In Vitro Tests of the Adherence* of Staphylococcus saprophyticus *and* Staphylococcus epidermidis *to Epithelial Cells from Different Sites of Two Healthy Males and Five Healthy Females*

Sex of persons studied	Cells used	Bacteria added								Bacteria not added			
		S saprophyticus				*S epidermidis*							
		No of cells to which bacteria had adhered/total no of cells studied	No of bacteria adhered/cell			No of cells to which bacteria had adhered/total no of cells studied	No of bacteria adhered/cell			No of cells to which bacteria had adhered/total no of cells studied	No of bacteria adhered/cell		
			M	Range	S E		M	Range	S E		M	Range	S E
Females	Periurethral	261/300	20 8	0–189	1 51	183/250	10 2	0–113	0 99	11/350	0 13	0–12	0 05
	Urothelial	269/300	14 85	0–123	1 11	145/250	5 18	0–79	0 72	8/350	0 05	0–4	0 02
	Skin	213/300	11 0	0–136	1 62	185/250	8 52	0–98	0 78	31/265	0 32	0–7	0 07
	Buccal	216/300	3 9	0–17	0 33	209/250	8 94	0–62	0 65	40/300	0 70	0–9	0 30
Males	Urothelial	13/121	20 7	0–151	2 76	100/65	5 44	0–49	0 91	5/25	0 23	0–16	0 14
	Skin	32/47	5 32	0–41	1 13	23/50	5 90	0–64	1 78	0/41	0	0–0	0
	Buccal	82/150	1 82	0–20	0 24	83/100	5 63	0–30	0 60	6/150	0 087	0–3	0 037

TABLE 2 *In Vitro Tests of the Adherence* of Staphylococcus saprophyticus *and* Staphylococcus epidermidis *to Epithelial Cells from Different Sites of Healthy Sow and Boar*

Sex of pigs studied	Cells used	Bacteria added								Bacteria not added			
		S saprophyticus				*S epidermidis*							
		No of cells to which bacteria had adhered/total no of cells studied	No of bacteria adhered/cell			No of cells to which bacteria had adhered/total no of cells studied	No of bacteria adhered/cell			No of cells to which bacteria had adhered/total no of cells studied	No of bacteria adhered/cell		
			M	Range	S E		M	Range	S E		M	Range	S E
Sow	Urothelial	41/50	13 5	0–69	2 11	39/50	10 6	0–66	2 23	0/50	0	0–0	0
	Skin	51/51	19 0	3–96	2 29	38/50	8 04	0–67	1 68	18/50	2 44	0–26	0 69
	Buccal	48/50	15 5	0–42	1 58	47/50	15 8	0–52	1 95	34/50	4 36	0–26	0 75
Boar	Urothelial	47/50	8 7	0–29	1 19	40/50	9 74	0–66	2 15	0/50	0	0–0	0
	Skin	47/50	12 2	0–58	1 81	31/50	6 22	0–53	1 37	0/50	0	0–0	0
	Buccal	50/51	16 9	1–66	1 93	41/50	7 4	0–37	1 15	18/50	1 11	0–9	0 29

Stud es of Ur ne Sed ments of Patients with UTI Caused by S saprophyticus

Voided urine specimens from two women with signs of acute cystourethritis and which contained $>10^5$ bacteria 1 ml^{-1} of *S saprophyticus* was centrifuged at 3 000 rev/min for 10 minutes The deposits were washed in PBS and fixed for 24 hours in a solution of 2 5 per cent (v/w) glutaraldehyde in 0 1 M PBS with an osmolarity of 499 mOsmol Further preparations of the urine sediments for scanning electron microscopy (SEM) were made as earlier described (17) The preparations were transferred to Nucleopore® filters (Nucleopore Corporation US) The urine sediments stained according to *Sternheimer & Malbin* (25) were also studied as »wet smears« under a light microscope (× 720)

Sta stical Methods

The statistical methods used were t test analysis of variance including Duncan s multiple range test (6) and Bartholomew s test for ordered alternatives (3) Due to an extreme skewness in the distribution of the number of adhered bacteria per cell in each experiment all calculations were based on logarithmic transformed values to give log normal distributions (4)

RESULTS

Adherence Test

Human epithelial cells The adherence of *S saprophyticus* and *S epidermidis* to urothelial cells (recovered from voided urine specimens) and to periurethral skin and buccal cells of the two males and the five females studied are given in Table 1 *S saprophyticus* adhered by significantly ($p < 0.05$) higher numbers to the periurethral and exfoliated urothelial cells than to the skin and buccal cells respectively *S epidermidis* adhered by significantly ($p < 0.05$) higher numbers to the periurethral cells than to the other types of cells tested *S saprophyticus* adhered by significantly ($p < 0.001$) higher numbers to both periurethral and exfoliated urothelial cells than did *S epidermidis* No difference was found between the two species as to their adherence to skin cells On the other hand *S epidermidis* adhered by significantly ($p < 0.001$) higher numbers to the buccal cells than did *S saprophyticus*

No significant difference was found in the number of adhered bacteria when using exfoliated urothelial cells from the males and females neither when testing *S saprophyticus* nor *S epidermidis* However *S saprophyticus* adhered by significantly higher numbers to skin ($p < 0.001$) and buccal ($p < 0.001$) cells from the females than from the males Such a sex difference was also found when testing the adherence of *S epidermidis* to buccal cells ($p < 0.001$)

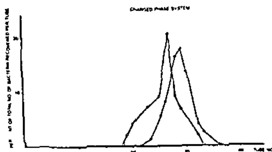


Fig 1 Counter-current distribution of one hemagglutinating (O——O) and one non hemagglutinating (●——●) strain of *S saprophyticus* in a charged two-polymer aqueous phase system

Porcine epithelial cells The results of the adherence test of *S saprophyticus* and *S epidermidis* to the different types of epithelial cells collected from a boar and a sow are given in Table 2 There was no significant difference in the adherence of *S saprophyticus* to any of the cells tested *S saprophyticus* and *S epidermidis* did not show any significant difference in their capacity to adhere to the exfoliated cells from the urinary tract of the boar and the sow On the other hand *S epidermidis* adhered by significantly ($p < 0.01$) lower numbers to the skin cells of both pigs than did *S saprophyticus* while no such difference was found when testing exfoliated cells from the urinary tract

Hemagglutination Tests

One of the two strains of *S saprophyticus* caused hemagglutination but none of the three strains of *S epidermidis*

Partition Tests

Both the hemagglutinating and the non hemagglutinating strain of *S saprophyticus* exhibited a preference for the top phase in the charged system i e both strains were negatively charged and had approximately the same charge-density (Fig 1) Their partition ratios (G) being 2 45 and 3 22 respectively calculated by the following formula $G = W_{max} / N - W_{max}$ (W_{max} represents the number of the tube with the highest concentration of bacteria and N the number of transfers) In the non charged phase system (in which the PEG rich top phase attracts particles with a hydrophobic surface character) both strains were only to a minor degree attracted by the top phase Their partition ratios were 0 12 and 0 23 respectively (Fig 2)

For comparison three strains of *S epidermidis* were tested in the same phase systems All three strains were attracted by the top phase in the

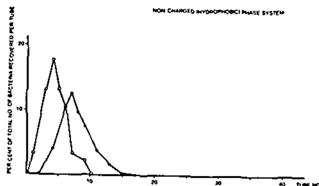


Fig 2 Counter-current distribution of the same strains as depicted in *Fig 1* in a non-charged (hydrophobic) two-polymer, aqueous phase system

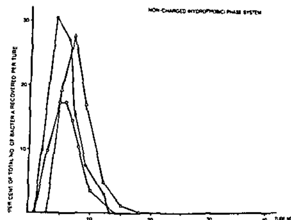


Fig 4 Counter-current distribution of the same three strains of *S epidermidis* as depicted in *Fig 3* in a non charged (hydrophobic) two-polymer, aqueous phase system

charged phase-system but to a lesser extent then the strains of *S saprophyticus* The partition ratio for the *S epidermidis* strains were 0 9, 0 15 and 0 19, respectively (Fig 3) When studied in the non-charged phase system all the latter three strains were as poorly attracted by a top-phase as S *saprophyticus* Their partition ratios in this system were 0 23, 0 16 and 0 16, respectively (Fig 4) Thus the surface of the three examined strains of *S epidermidis* was negatively charged, but had a lower charge density at pH 7 2 than the two tested strains of *S saprophyticus* All five staphylococcal strains had a poor liability for hydrophobic interaction

Studies of Urine Sediments

Light microscopic examination of the urine sediments of patients with UTI caused by *S saprophyticus* revealed urothelial cells and casts to which numerous single cocci or clusters of cocci had adhered The adhering and cluster-forming tendency of *S saprophyticus* was also visualized in SEM preparations (Fig 5)

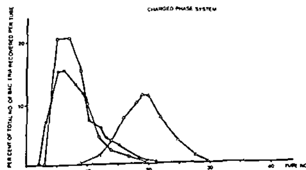


Fig 3 Counter-current distribution of three different strains of *S epidermidis* in a charged two polymer, aqueous phase system

DISCUSSION

The ability of bacteria to adhere to the mucosal lining of the urinary tract seems to be *one* prerequisite for the development of UTI (26) In *in vitro* experiments, the capacity of different bacterial species to adhere to a given type of epithelial cells may vary This is also true for a given bacterial species, when testing cells of different origin (18) The differential capacity to adhere to a given type of epithelial cell may be one explanation of the organ tropism exhibited by certain bacteria

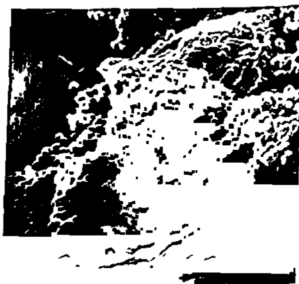


Fig 5 Scanning electron micrograph of an exfoliated squamous epithelial cell collected on a Nucleopore® filter, obtained from urine of a women with a UTI caused by *S saprophyticus* The epithelial cell surface is almost covered with cocci, adhering to the cell and to one another (× 2,180)

In contrast to other establishment urinary tract pathogens of man *S saprophyticus* is known to cause clinical infection only in the urinary tract

In the *in vitro* tests *S saprophyticus* adhered by significantly higher number to human urothelial and periurethral cells than to skin and buccal cells We also found *S saprophyticus* to have a significantly higher capacity to adhere to exfoliated cells from the urogenital tract than *S epidermidis* UTI with *S saprophyticus* often seems to occur in that group of approximately 10–15 per cent of all women in whom recurrent UTI can be demonstrated (9) while *S epidermidis* is but rarely a cause of UTI in this group of women The latter organism is known to occur in the indigenous flora at each site from which we collected cells for our adherence experiments

Coagulase negative novobiocin resistant staphylococci e g *S saprophyticus* are often found in the skin flora of laboratory and domestic animals including pigs (16) where they seem to predominate among the coagulase negative staphylococci However on the human skin novobiocin-sensitive strains e g *S epidermidis* predominate among the coagulase negative staphylococci Coagulase negative novobiocin resistant staphylococci are *not* known as urinary tract pathogens in the pig while in man such staphylococci viz *S saprophyticus* are established pathogens at this site (9 15)

In the *in vitro* adherence tests using porcine epithelial cells *S saprophyticus* adhered by significantly higher numbers to skin cells than did *S epidermidis* while there was no difference between these bacterial species when testing cells from the urogenital tract This was in contrast to when using corresponding cells from humans The results of our *in vitro* adherence tests thus suggest one possible mechanism by which the tropism of *S saprophyticus* for the urinary tract in man can be explained Its marked capacity to adhere to urogenital epithelial cells may also express a pathogenecity factor of the organism at this particular site

In accordance with the results of the in vitro adherence tests urine sediments of patients with UTI caused by *S saprophyticus* showed numerous cocci adhered to epithelial cells Such cells covered by cocci were found both in voided urine and in urine specimens obtained by suprapubic bladder aspiration The light microscopic appearance of urine sediments of patients with UTI caused by *S saprophyticus* were highly characteristic In our experience the finding of a urinary sediment with these characteristics is with a high degree of accuracy consistant with a UTI caused by *S saprophyticus*

The interaction between cells is depending on their physico-chemical surface properties (29) The colonization of porcine intestinal epithelium by enteropathogenic *Escherichia coli* is exaggregated in strains carrying K 88 antigen (11 12) Such strains also cause mannose resistant hemagglutination of guinea pig erythrocytes (12) A prerequisite for porcine enteropathogenic *E coli* to attach to intestinal epithelial seems to be the presence of pilus like adhesins being or being closely related to the K 88 antigen (7 10)

The presence of K 88 antigen will provide the cell with a liability for hydrophobic interaction (22) In addition to hydrophobic interaction liability a negative surface charge seems to favour the association of certain enterobacteria to intestinal epithelial cells in the mouse (19 23 24)

In only a few bacterial species viz *Salmonella typhimurium* both charge and hydrophobic/hydrophilic interaction liability has been studied in relation to the interaction of the bacterium with urothelial cells (27) This study indicates that negative surface charge combined with a hydrophilic surface favour the adherence of bacteria to such cells

The present study suggested *S saprophyticus* to have surface properties rendering it more liable to interact with urothelial cells than *S epidermidis* despite *S saprophyticus* having a higher surface charge-density

However surface properties such as charge charge-density and hydrophobic/hydrophilic interaction liability do not fully explain all bacteria-cell interaction phenomena as for example these properties differed only to a very limited extent between the strain of *S saprophyticus* with hemagglutinating capability and the strain that lacked the capacity of such cell interaction Though critizised (5) it has been claimed that the second stage of hemagglutination is facilitated by a reduced negative surface charge (20) Our study does not present convicing evidence for such an assumption as surface charge density only to a minor degree differed between the hemagglutinating and the non hemagglutinating strain of *S saprophyticus*

We thank Professor *P Å Albertsson* and co workers at the Department of Biochemistry Chemical Center Lund for providing facilities to run the CCD-tests

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Acta path microbiol scand Sect. B 87 329–336 1979

CROSS-REACTIONS BETWEEN *STAPHYLOCOCCUS AUREUS* AND FIFTEEN OTHER BACTERIAL SPECIES

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Schiøtz, P O Høiby N & Hertz J B Cross reactions between *Staphylococcus aureus* and fifteen other bacterial species Acta path microbiol scand Sect. B 87 329–336 1979

Fifty five different antigens were demonstrated in an antigen preparation obtained by sonication of a protein A free strain of *Staphylococcus aureus* using crossed immunoelectrophoresis and antiserum obtained from rabbits The antigens were characterized by absorption experiments with formalin killed bacterial cells temperature resistance and protein and polysaccharide staining One of the antigens showed reaction of identity with a preparation of teichoic acid from *S aureus* Using this reference system cross reactions between the *S aureus* reference strain and *S aureus* strains from the four phage groups as well as 15 other bacterial species were studied by various quantitative immunoelectrophoretic methods The *S aureus* antigens from the four phage groups showed almost 100% cross reactivity for all antigens while antigens from nine other bacterial species cross reacted to varying degrees with one to four *S aureus* antigens All of 12 *S aureus* antigens cross reacted with the four *S epidermidis* biotypes

Key words *Staphylococcus aureus* crossed immunoelectrophoresis cross reactivity antigen

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The antigenic structure of *Staphylococcus aureus* has been investigated by several methods (1 4 5 6 7 18 24 25) However analysis of the antigenic components of *S aureus* carried out by quantitative immunoelectrophoretic methods has not been reported previously These methods have been employed to investigate the antigenic composition of a number of Gram negative bacteria (*Pseudomonas aeruginosa Bordetella pertussis Neisseria meningitidis* and *Haemophilus influenzae*) (10 14 16 26) In this way several antigens have been found which cross react with other Gram negative bacterial species However cross reactions with Gram positive bacteria were sometimes seen

The aim of the present study was to investigate the antigens of a common Gram positive bacteria *S aureus* by quantitative immunoelectrophoretic techniques and to examine the cross reactivity of the *S aureus* antigens with other bacteria

MATERIALS AND METHODS

Preparation of a Polyvalent Reference S aureus *antigen (Ag)*

S aureus (E 1369 coagulase positive phage type

... for 24 h (11) The bacteria were scraped off the plates washed three times and disintegrated by sonication for 3 × 5 min at 20 000 Hz/s using Rapidis 300 19 mm probe with 9 5 mm tip cooled with iced water as described previously (14) The disintegrates were centrifuged at 48 000 × *g* for 1 h at 4 °C and the supernatant stored in

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the immunization and bleeding schedule of *Harboe & Ingild* (9) Each animal received 100 µl *S aureus* Ag per injection IgG and IgA were purified and concentrated (9). Immunoglobulins from bleeding 4–15 were pooled since they showed similar antibody patterns The final preparation contained 29 0 g protein/l as determined by refractometry

Control experiments running sonicated culture medium against *S aureus* Ab in crossed immunoelectrophoresis with intermediate gel showed that none of the antibodies precipitated with culture medium components

Preparation of Antigens from Other Strains of Staphylococci and Other Bacterial Species

The origin of most of the bacteria shown in Table I has been described previously (14)

The *S aureus* strains and the *S epidermidis* strains were isolated from blood cultures The following *S aureus* strains were isolated *S aureus* phage type 52/52A/80/81 *S aureus* phage type 3A *S aureus* phage type 6/47/53/75/93 *S aureus* phage type 42D and *S aureus* non typeable All these strains were protein A positive The antigens were obtained by sonication as described previously (14) The colloid concentrations of these antigens were 3 2–18 9 g/l (refractometry using human IgG as standard)

Immunoelectrophoretic Methods

Immunoelectrophoretic analysis of *S aureus* Ag was performed by crossed immunoelectrophoresis according to *Weeke* (28) as described previously (14 15) Optimal resolution was obtained if 5 µl *S aureus* Ag was [illegible]

[illegible] blue (proteins) and periodic acid Schiff (polysacchar des) (27)

Crossed immuno-affinoelectrophoresis was carried out as described above but with 150 µg concanavalin A (con A) sepharose/cm² or 150 µg free con A/cm² in the intermediate gel The con A sepharose and con A were purchased from Pharmacia Fine Chemicals Uppsala Sweden

Antigens from each of the bacteria were compared with the *S aureus* Ag by means of quantitative immunoelectrophoresis This was also done with the preparation of teichoic acid Each antigen preparation was run 1) *in crossed immunoelectrophoresis* (12) according to *Weeke* (28) against *S aureus* Ab 2) in *tandem crossed immunoelectrophoresis* (14) according to *Krøll* (19) with *S aureus* Ag against *S aureus* Ab 3) *in crossed line electrophoresis* (13 14) according to *Krøll*

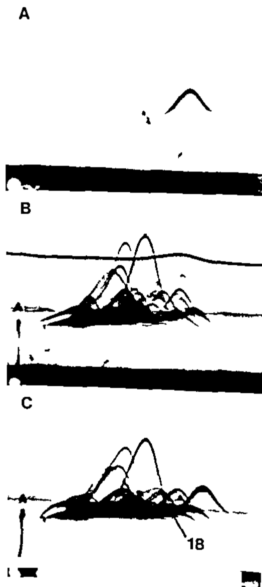


Fig 2 A–C Comparison of *Staphylococcus aureus* cell wall teichoic acid with *S aureus* antigens (*S aureus* Ag) using rabbit anti-*S aureus* antiserum (*S aureus* Ab)
A Crossed immunoelectrophoresis of 5 µl teichoic acid against *S aureus* Ab Saline in the intermediate gel One precipitate is visible
B Crossed line immunoelectrophoresis of *S aureus* Ag against *S aureus* Ab with teichoic acid in the intermediate gel (absorption of antibodies in situ) One of the precipitates of the reference pattern has been absorbed (No 18) and can be seen as a small arc on the precipitation line above the reference pattern Compare with I A & I B
C Tandem-crossed immunoelectrophoresis of *S aureus* Ag in the left well and teichoic acid in the right well against *S aureus* Ab Saline in the intermediate gel Reaction of identity between the teichoic acid and *S aureus* antigen No 18 is seen (arrow) Compare with I A

Technical data First dimension electrophoresis anode to the right. Second dimension electrophoresis anode at the top

Fig 1A

0 2 0 4 0 6 0.8 1 0 2 0

1 B



Fig 1 A & B A Drawing of the *Staphylococcus aureus* antigen/antibody reference pattern with enumeration of the precipitates seen on the corresponding immunoplate (B) An arbitrary scale of migration velocity relative to human serum albumin (1 0) is indicated below

small aliquots at −30 °C until use as antigen preparation for immunization of rabbits or electrophoresis experiments The colloid concentration as measured by refractometry using human IgG as standard was 5 8 g/l A protein A free *S aureus* was used because *S aureus* containing protein A are known to induce antibodies against hidden determinants of autologous rabbit IgG (21)

Immunoelectrophoresis according to *Grabar & Williams* (8) as described previously (17) of 10 µl *S aureus* Ag against 200 µl *S aureus* standard antibody showed that all the antigens migrated towards the anode

Aliquots of *S aureus* Ag were autoclaved at 120 °C for 30 min and then run in crossed immunoelectrophoresis with intermediate gel against *S aureus* standard antibody Absorption experiments were carried out with formalin killed *S aureus* cells (1 % formalin 37 °C 1 h) as described previously (13)

Preparation of Teichoic Acid

The bacteria were cultured and disrupted (sonication 5 min × 2) as described The 10 000 × *g* sediment of disrupted cells was used to prepare teichoic acid according to *Morse* (23) The colloid concentration of the final preparation of teichoic acid in isotonic phosphate buffer (pH = 7 0) was 2 1 g/l (refractometry using IgG as standard)

Preparation of a Polyvalent Rabbit Reference S aureus *Antiserum (Ab)*

Six rabbits were immunized intradermally with *S aureus* Ag in Freunds incomplete adjuvant following

A



1)

B

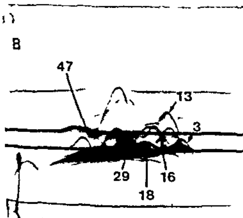


Fig 3 A & B Comparison of antigens from Staphylococcus epidermidis biotype IV with *Staphylococcus aureus* antigens (S aureus Ag) using rabbit anti *S aureus* antiserum (*S aureus* Ab)

A Crossed immunoelectrophoresis of *S epidermidis* antigens against *S aureus* Ab Saline in the intermediate gel Thirteen distinct precipitates are visible

B Crossed line immunoelectrophoresis of *S aureus* Ag against *S aureus* Ab with *S epidermidis* antigens in the intermediate gel (absorption of antibodies *in situ*) Six of the precipitates of the reference pattern have been completely absorbed (Nos 21 24 36 41 46 and 52) and six of the precipitates of the reference pattern have increased in area (Nos 3 13 16 18 29 and 47 indicated by arrows) Control reference pattern of *S aureus* Ag against *S aureus* Ab is seen in Fig 4A Thus one of the *S epidermidis* precipitates (A) did not cross react with *S aureus* Technical data as Fig 2

anode and the migration velocity of the various antigens is indicated in relation to human serum albumin (Fig 1B) 55 precipitates could be detected in the reference antigen/antibody pattern 49 were regularly visible while the remaining precipitates could only be detected when the Ag/Ab ratio was changed

All preimmunization sera from the six rabbits contained antibodies corresponding to precipitate No 46

The autoclaving of the *S aureus* Ag at 120 °C for 30 minutes caused disappearence of all the precipitates except Nos 29, 46 and 52 The absorption experiments with whole formaline-killed [illegible] these cells could absorb

[illegible]

[illegible]

aureus Ag/ *S aureus* Ab reference pattern were stained by the periodic acid Schiff reaction (polysaccharides)

Antigen No 18 was identical with the teichoic acid (see Fig 2 A-C), and in the crossed immunoaffinoelectrophoresis with con A and con-A Sepharose antigen No 18 was the only antigen that showed affinity for con-A, while con A Sepharose did not react with any of the *S aureus* antigens

Cross-reactions between the five different *S aureus* strains and the *S aureus* reference Ag showed 100 % cross-reactivity for all antigens with all strains except for antigen No 52 which cross-reacted only 25–50 % with the *S aureus* of phage group I

Table 1 shows the results of the comparison of antigens from 15 different bacterial species with the *S aureus* Ag/*S aureus* Ab reference system All of 12 antigens were found to cross-react with *S epidermidis* biotypes I–IV (see Table 1 and Fig 3A–B) One or more of antigens Nos 13, 24 36, 41 and 52 were cross reactive with nine different bacterial species both Gram positive and Gram negative an example is given in Fig 4 A–D for details see Table 1

DISCUSSION

Our results show that 55 antigens can be demonstrated in a protein A-free *S aureus* sonicate employing sera from immunized rabbits The findings are in accordance with the antigenic heterogeneity demonstrated for other bacteria (*P aeruginosa B pertussis N meningitidis* and *H influenzae*) when similar methods are used (10, 14 16 26)

The investigations of the *S aureus* Ag show that antigen No 18 is identical with teichoic acid This is demonstrated by the reaction of identity found by electrophoresis, but is also supported by the fact that antigen No 18 is among the antigens that stain positively for polysaccharides (antigens Nos 18, 29, 46 52) Furthermore, this is in accordance with our finding of antigen No 18 as one of the two surface antigens (Nos 18 and 24) Binding of antigen No 18 to the lectin con A shows that it contains α D-glucopyranoside substituents, and that it has more than one binding site to con A since there was no interaction between antigen No 18 and con A

TABLE 1 *Cross-reactions between* Staphylococcus aureus *and Other Bacterial Species The Numbers Signify the Cross-reactive Antigens in the Reference System The Number of Strains Tested and the Group Type or Collection Numbers are Given in Brackets*

SPECIES	Cross reactive antigens and percentage of cross reactivity			
	100%	100% >– ≧75%	75% >– ≧50%	50% >– ≧25%
Staphylococcus epidermidis biotype I, (1)	21–24–36 41–46	16–18–29 47–52	3–13	
Staphylococcus epidermidis biotype II, (1)	21–24–36 41–46–52	3–18–29	13–16–47	
Staphylococcus epidermidis biotype III, (1)	24–36 41–46	3–13–16–18 21–29–52	47	
Staphylococcus epidermidis biotype IV, (1)	21–24–36 41–46–52	3–13–18–29	16–47	
Streptococcus faecalis (1)	41	24–52		
Streptococcus pyogenes group A (1)		52	24	13
Bacillus cereus var mycoides (ATCC 11778)(1)	52	3–47	24	
Bacillus subtilis (1)		36–41	24	
Listeria monocytogenes (1)				
Corynebacterium species (1)				
Clostridium welchii (1)				
Neisseria meningitidis 1, group A)				
Escherichia coli (1,021 H27)			24	
Klebsiella pneumonia (1 type 35)			24	
Proteus mirabilis (1)	52		24–41	
Pseudomonas aeruginosa (4 0 groups 3, 5, 6 11)			24	
Bordetella pertussis 4 st 3803, 3825 3843, 3860)				
Haemophilus influenzae (capsulate type b biotype I)				

(20) with the antigen in question included in an intermediate gel between first and second dimension electrophoresis of *S aureus* Ag against *S aureus* Ab (absorption of antibodies *in situ*)

The analytical electrophoresis series were repeated at least twice using different antigen/antibody ratios (14) The percentage of antibodies absorbed *in situ* in the crossed line immunoelectrophoresis was estimated by comparison with sets of four standard plates containing 25%, 50% 75% and 100% respectively of the original concentration of *S aureus* Ab as described previously (14) The increase in enclosed area by a given precipitate after absorption *in situ* can be expressed as 100%, 100%>– ≧75% 75% >– ≧50% 50% >– ≧25% absorption of antibodies against the antigen in question Absorption of less than 25% was considered insignificant, bearing in mind the analytical variation of the present method (13 14) The first dimension electrophoresis of the antigens was run with 2 µl or 1 µl and appropriate combinations of these volumes in the same well (addition) or in the wells of the tandem crossed immunoelectrophoresis The intermediate gel of crossed-line immunoelectrophoresis contained 20 µl or 40 µl of the antigen in question or saline as control and the reference gel contained *S aureus* Ab 20 µl/cm² *Immunoplates compared by these methods were always* run simultaneously All the antigens of *S aureus* Ag were stable at the mixing temperature of the agarose (40 °C)

RESULTS

Fig 1A and B show a reference plate with enumerated precipitates and a corresponding drawing The precipitates are numbered from the

could not confirm the presence of any cross reactivity between *S aureus* and *H influenzae* antigens in our investigation (23 Table 1) The reason for this discrepancy between our results and the results of *Argaman et al* (1) is not clear though different technical approaches might be involved On the basis of the present results the role of cross reacting antibodies in protection against *S aureus* infections seems to demand further studies on patients and even more so in the laboratory since the importance of humoral versus cellular immunity in protection against *S aureus* infections has not been definitely determined (4)

This work was supported by the *Danish Medical Research Council* (Project Nos 512-5267 6623 and 8115) The *National Association for the Fight against Tuberculosis* the *Bergsøe Foundation* and the *National Danish Association against Cystic Fibrosis* We thank *Anni Bethien* and *Elinor Ward Petersen* for skilful technical assistance

Dr Kirsten Rosendal Statens Seruminstitut has kindly provided the strains of Staphylococcus and has carried out the biotyping and phage typing of the strains

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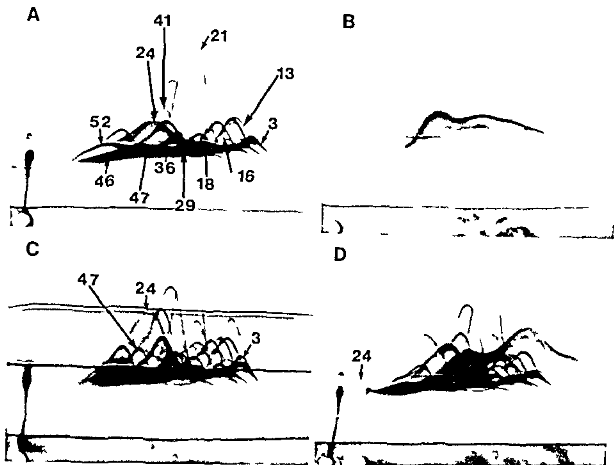


Fig 4 A–D Comparison of antigens from *Bacillus cereus* var *mycoides* with *Staphylococcus aureus* antigens (*S aureus* Ag) using rabbit anti *S aureus* antiserum (*S aureus* Ab)
A Crossed immunoelectrophoresis of *S aureus* Ag against *S aureus* Ab Saline in the intermediate gel Precipitates Nos 3 24 47 and 52 are among the precipitates indicated by arrows
B Crossed immunoelectrophoresis of *B cereus* antigens against *S aureus* Ab Saline in the intermediate gel Seven distinct precipitates are visible
C Crossed line immunoelectrophoresis of *S aureus* Ag against *S aureus* Ab with *B cereus* antigens in the intermediate gel (absorption of antibodies *in situ*) One of the precipitates of the reference pattern has been completely absorbed (52) and three of the precipitates of the reference pattern have increased in area (Nos 3 24 and 47 indicated by arrows) Compare with 4 A
D Tandem crossed immunoelectrophoresis of *S aureus* Ag in the left well and *B cereus* antigen in the right well against *S aureus* Ab Saline in the intermediate gel Reaction of identity between one of the *B cereus* antigens and *S aureus* antigen No 24 (arrow) can be seen Compare with 4 A Thus three of the *B cereus* precipitates (B) did not cross react with *S aureus* Techn cal data as Fig 2

Sepharose (2 23) This is in agreement with the structure of wall teichoic acid in *S aureus* which contains polymers of either glycerol or ribitol phosphates substituted with various residues (3) One of these residues is N acetyl D glucosamine which is an α D glucopyranoside

Extensive cross reactivity was found between the different *S aureus* strains which corresponds to previous results with different bacteria where strains of the same species were found to cross react extensively (10 14 16 23) Correspondingly we have also found considerable (though less) cross reactivity between different species of the same genus – in this case the four biotypes of *S epidermidis* – compared with our *S aureus* Ag/ *S aureus* Ab reference system Only few antigens cross react with more remotely related species both Gram positive and Gram negative bacteria

Cross reacting antibodies between Gram positive bacteria as well as between Gram positive (including *S aureus*) and Gram negative bacteria have been demonstrated previously (1 29) It was suggested that this was due to the polyribitol phosphate of teichoic acids which should share antigenic determinants with the capsular polysacc haride of *H influen-ae* type b (1) However we

Acta path microbiol scand Sect. B 87 337–344 1979

CROSS-REACTIONS BETWEEN *HAEMOPHILUS INFLUENZAE* AND NINETEEN OTHER BACTERIAL SPECIES

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Schiøtz P O Høiby N & Hertz J B Cross reactions between *Haemophilus influenzae* and nineteen other bacterial species Acta path microbiol scand Sect. B 87 337–344 1979

Forty-one different antigens were demonstrated in an antigen preparation obtained by sonication of a *Haemophilus influenzae* (H influenzae) type b strain using crossed immunoelectrophoresis and antiserum obtained from rabbits Antigens were characterized by absorption experiments with whole heat killed bacteria temperature resistance and protein and polysaccharide staining Cross reactions between *H influenzae* type b and 19 other bacterial species were studied by various quantitative immunoelectrophoretic methods using the reference system A non-capsulated *H influenzae* cross reacted extensively (41 antigens) with *H influenzae* type b and *Haemophilus parainfluenzae* and *Haemophilus haemolyticus* showed considerable cross reactivity with *H influenzae* type b (26 and 32 antigens respectively) while antigens from eight other bacterial species cross reacted to varying degrees with one to five *H influenzae* antigens

Key words *Haemophilus influenzae* antigens crossed immunoelectrophoresis cross reactivity

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Many studies have been concerned with the antigenic composition of *Haemophilus influenzae* and the biological significance of its different antigens (3 4 5 6 30 31) Analysis of *H influenzae* antigens carried out by quantitative immunoelectrophoretic methods has previously only been reported with regard to the capsular antigen of *H influenzae* type b (6) In the present study we therefore also investigated other antigens of *H influenzae* by means of quantitative immunoelectrophoretic methods and examined the cross reactivity of the *H influenzae* antigens with other bacterial species using the methods employed previously on studies of the antigenic structure and cross reactivity of *Pseudomonas aeruginosa* *Neisseria meningitidis* *Bordetella pertussis* and *Staphylococcus aureus* (13 19 23 32)

MATERIALS AND METHODS

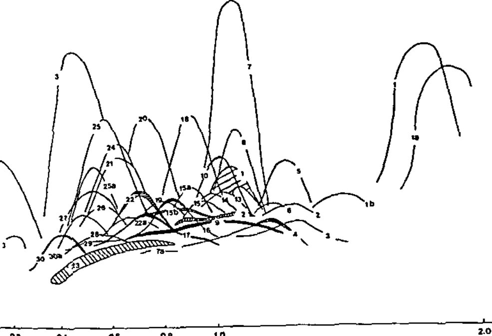
Preparation of a Polyvalent Reference H influenzae *antigen (H inf Ag)*

A capsulate *H influenzae* type b (designated N in the culture collection of our laboratory (biotype I according to *Kilian* (25)) isolated from the spinal fluid of a patient [illegible]

[illegible] up cooled with iced water [illegible] described [illegible]

[illegible] use as antigen preparation for immunization of rabbits and electrophoresis experiments

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Drawing of the *Haemoph lus influen ae* antigen/ *Haemophilus influen ae* antibody reference pattern with s on of the precip tates An arb trary scale of migration velocity relative to human serum albumin (1 0) is d below The corresponding immunoplate is seen in Fig 2A

rated from the anode and the migration y of the various antigens is indicated in n to huma serum albumin (Fig 1 and 2A)

immunization sera from the six rabbits ned antibodies corresponding to precipitates 4 9 23 and 29

ugens Nos 4 9 and 23 were the only ones in inf Ag/H inf Ab reference system which stained by both the periodic acid Schiff reac polysaccharides) and by Coomassie Brilliant (proteins) which also stained the remaining p tates Autoclaving of the H inf Ag at C for 30 minutes destroyed the antigenicity of e antigens except Nos 9 21 and 23 However morphology of these precipitates changed edly and part of precipitate No 9 disappeared absorption experiments with heat killed whole rial cells showed that these cells could absorb antibodies against antigens Nos 9 and 21

ne commercial *H influen ae* capsular polysacc de vaccine showed only one precipitate with mercial *H influen ae* capsular antibody Reac of 100% identity was found between this ip tate and a precipitate between H inf Ag and mercial *H influen ae* capsular antibody This ipitate was however not present in the H inf /H inf Ab reference pattern

The results of comparison of antigens from 19 different bacterial species with the H inf Ag/H inf Ab reference system are given in Table 1 Fig 2 A D shows a comparison of *Escherichia coli* and *H influen ae* antigens One antigen (No 4) was found in nine different bacterial species all of which were Gram negative Furthermore antigens Nos 7 8 10 16 21 22 24 and 25 were shown to be cross reactive mostly with Gram negative bacteria (for details see Table 1) Cross reactions between anti gens of *P aeruginosa B pertussis* and *N meningiti dis* have been described previously (13 19 23) and the numbers of corresponding antigens within the different reference patterns have been outlined

	Antigen numbers	
H influen ae	4	16
P aeruginosa	10	24
B pertuss s	11	28
N men ng t d s	19	38

The colloid concentration, measured by refractometry using human IgG as standard, was 4 9 g/l

Immunoelectrophoresis, according to *Grabar & Williams* (9) as described previously (24), of 10 µl H inf-Ag against 200 µl *H influenzae* standard antibody (H inf-Ab) showed that most of the antigens migrated towards the anode However, at least four antigens migrated towards the catode and therefore escaped detection in the present study, where the conventional crossed immunoelectrophoresis used detected only anodically migrating antigens

Aliquots of H inf-Ag were also autoclaved at 120 °C for 30 min and then run in crossed immunoelectrophoresis with intermediate gel against H inf-Ab

Absorption experiments were carried out with whole, heatkilled *H influenzae* bacteria, as described previously (21)

Preparation of a Polyvalent Rabbit Reference H inf-Ab

Six rabbits were immunized intradermally with H inf-Ag in Freunds incomplete adjuvant (1 1) following the immunization and bleeding schedule of *Harboe & Ingild* (11) Each animal received 100 µl H inf-Ag per injection IgG and IgA were purified and concentrated (11) Immunoglobulins from bleedings 1–3 were pooled, since electrophoretic analysis revealed similar antibody patterns This preparation contained 32 0 g protein/l as determined by refractometry and was used in the present work Control electrophoresis experiments running sonicated culture medium against H inf-Ab showed that none of the antibodies precipitated with culture medium components

Preparation of Antigens from 19 other Bacterial Species

The origin of most of the bacterial strains examined (recorded in Table 1) and the preparation of antigens by sonication have been described previously (19) *Haemophilus parainfluenzae* (biotype III) (designated bc in the culture collection of our laboratory) and *Haemophilus haemolyticus* (designated NH in the culture collection of our laboratory) were isolated from clinical specimens from the upper respiratory tract received from the Department of Diagnostic Bacteriology Statens Serum institut The colloid concentrations of these antigens were 3 2–18 9 g/l (refractometry using human IgG as standard)

H influenzae *Capsular Polysaccharide*

A commercial vaccine preparation of capsular polysaccharide of *H influenzae* type b (20 µg/ml) (from Merck, Sharp & Dohme Rahway, New Jersey, USA) and a commercial antiserum raised against *H influenzae* type b (Statens Seruminstitut Copenhagen, Denmark) were employed in the experiments designed to identify a precipitate in the H inf-Ag/H inf-Ab pattern representing the capsular polysaccharide

Immunoelectrophoretic methods

Immunoelectrophoretic analysis of H inf-Ag was performed by crossed immunoelectrophoresis on 5 × 5 cm glass plates according to *Weeke* (34) as described previously (19, 20) Optimal resolution was obtained if 2 µl H inf-Ag was employed in the first dimension electrophoresis and 20 µl H inf-Ab/cm^2 in the second dimension gel The precipitates were stained with Coomassie Brilliant Blue (proteins) and periodic acid Schiff (polysaccharides) (33)

Antigens from each of the bacteria and the commercial *H influenzae* vaccine preparation of capsular polysaccharide were compared with the H inf-Ag by means of a series of quantitative immunoelectrophoreses Each antigen preparation was run (a) *in crossed immunoelectrophoresis* (18) according to *Weeke* (34) against H inf-Ab, (b) *in tandem-crossed immunoelectrophoresis* (19) according to *Krøll* (27) with H inf Ag (or added to the same first dimension well as H inf-Ag) against H inf-Ab, (c) *in crossed-line immunoelectrophoresis* according to *Krøll* (26), as described previously (19), with the antigen in question included in an intermediate gel between first and second dimension electrophoresis of H inf-Ag against H inf-Ab (absorption of antibodies *in situ*) The analytical series of electrophoreses were repeated at least twice using different antigen/antibody ratios (19) The percentage of antibodies absorbed *in situ* in the crossed line immunoelectrophoreses was estimated by comparison with sets of four standard plates containing 25%, 50%, 75% and 100%, respectively, of the original concentration of H inf-Ab, as described previously (19) The increase in enclosed area by a given precipitate after absorption *in situ* can be expressed as 100%, 100% >– ≧ 75% 75% > – ≧ 50%, 50% > – ≧ 25% absorption of antibodies against the antigen in question absorption of less than 25% was considered insignificant bearing in mind the analytical variation in the present method (19 22) The first dimension electrophoresis of the antigens was run with 2 µl or 1 µl and appropriate combinations of these volumes in the same well or in the wells of the tandem-crossed immunoelectrophoresis The intermediate gel of crossed line immunoelectrophoresis contained 20 µl or 40 µl of the antigen in question or saline as control and the reference gel contained H inf-Ag 20 µl/cm^2

The H inf-Ag was also compared with the commercial *H influenzae* vaccine preparation of capsular polysaccharide using the three electrophoretic techniques described but with commercial *H influenzae* capsular polysaccharide antiserum (20 µl/cm^2) in the second dimension gel

Immunoplates compared by these methods were always run simultaneously All the antigens of H inf-Ag were stable at the mixing temperature of the agarose (40 °C)

RESULTS

Forty-one immune precipitates were detectable in the H inf-Ag/H inf-Ab reference pattern, 39 of which were regularly visible The remaining precipitates could be detected only when the H inf-Ag/H inf-Ab ratio was changed The precipitates are

TABLE 1 *Cross reactions between* Haemophilus influenzae *and other Bacterial Species The Figures Signify the Cross reactive Antigens in the Reference System The Number of Strains Tested and the Group Type or Collection Numbers are given in Brackets*

SPECIES	Cross reactive antigens and percentage of cross reactivity			
	100%	100% > – ≧75%	75% > – ≧50%	50% > – ≧25%
Staphylococcus aureus (1 E1369 without protein A)				
Staphylococcus aureus (4 from each of the 4 phage groups with protein A)				
Staphylococcus epidermidis (1)				
Group G streptococcus	24			
Streptococcus faecalis (1)				
Streptococcus pneumonia (1 type 23 F)				
Bacillus cereus var mycoides (ATCC 11778)				
Bacillus subtil s (1)				
Corynebacterium species (1)				
Listeria monocytogenes (1)				
Clostridium welchii (1)	21			
Neisseria meningitidis (1 group A)		4	10–16	
Escherichia coli (1 021 H27)	21	4	22	8–10
Klebsiella pneumonia (1 type 35)	16–21	4		22
Proteus mirabilis (1)	4–25	21		
Pseudomonas maltophilia (1)				
Pseudomonas aeruginosa (4 O groups 3 5 6 11)	7	4–10–16		
Bordetella pertussis (4 st 3803 3825 3843 3860)		4–16		
Haemophilus influen ae (1 non capsulate biotype II)	39 antigens were 100% cross reactive a)			
Haemophilus parainfluen-ae (1 biotype III)	26 antigens were 100% cross reactive b)			
Haemophilus haemolyticus (1)	32 antigens were 100% cross reactive c)			

a) Antigens Nos 9 and 23 were 25–50% cross reactive
b) 15 antigens were not cross reactive (2 14 15 19 20 23 24 25 25a 26 27 30 30a 33 and one unidentifiable)
c) 9 antigens were not cross reactive (3 8 12 17 20 23 25 30 33)

DISCUSSION

The present study shows that 41 anodic migrating antigens and at least four catodic migrating antigens can be demonstrated in *H influenzae* sonicates employing sera from immunized rabbits This antigenic heterogenicity is in accordance with the findings with other bacteria (*P aeruginosa B pertussis N meningitidis* and *S aureus*) when similar methods are used (12 14 16)

The heat stability experiments and absorption experiments with heat killed whole bacterial cells show that of the thermostable antigens Nos 9 and 21 ... f the cell The heat stability of antigen No 23 combined with its polysaccharide content indicate that this antigen may be related to bacterial lipopolysaccharide

The experiments w th ...

that the type b capsular pol ...
antibody pr
H inf -Ab i
fact that *H influen ae* capsular polysaccharide is a poor imm ...

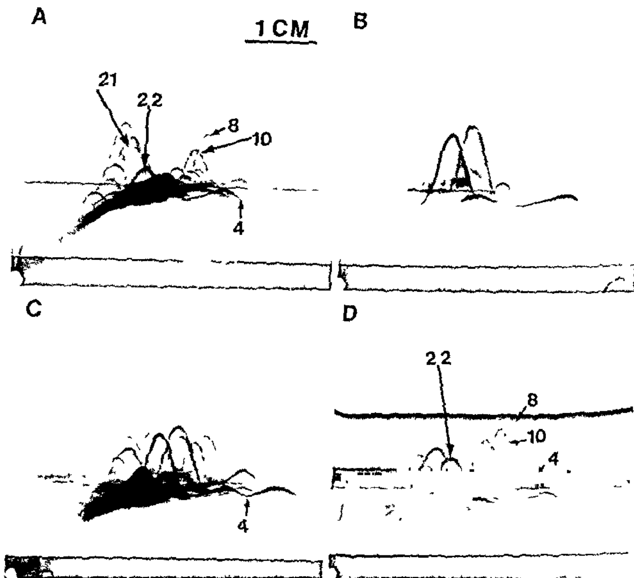
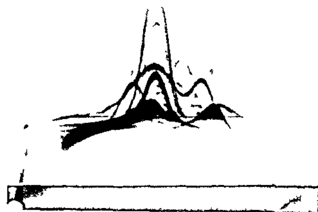


Fig 2 Comparison of antigens from *Escherichia coli* with *Haemophilus influenzae* antigens (H inf -Ag) using a pooled rabbit anti *H influenzae* antiserum (H inf Ab)
A Crossed immunoelectrophoresis of H inf -Ag against H inf -Ab Saline in the intermediate gel Precipitates Nos 4 8 10 21 and 22 are indicated by arrows
B Crossed immunoelectrophoresis of *E coli* antigens against H inf Ab Saline in the intermediate gel Nine distinct and two to three faint precipitates are visible
C Tandem crossed immunoelectrophoresis of H inf Ag (left well) and *E coli* antigens (right well) against H inf Ab Saline in the intermediate gel Reaction of identity between one of the *E coli* antigens (the anodic) and *H influenzae* antigen No 4 (arrow) is seen Compare with A and B
D Crossed-line immunoelectrophoresis of H inf -Ag against H inf -Ab with *E coli* antigens in the intermediate gel (absorption of antibodies *in situ*) One of the precipitates of the reference pattern has been completely absorbed (No 21) and four of the precipitates of the reference pattern have increased in area (Nos 4 8 10 and 22, indicated by arrows) Thus only five of the 11–12 precipitates visible in B are cross-reactive
Technical data First dimension electrophoresis anode to the right Second dimension electrophoresis anode at the top

Comparison with *H parainfluenzae* showed that 26 antigens cross-reacted with *H influenzae* 14 of the remaining antigens could be identified with certainty (see Figs 3A and B, Table 1) *H haemolyticus* cross-reacted with 32 *H influenzae* antigens, and the remaining 9 *H influenzae* antigens could all be identified (Table 1) Extensive cross-reactions were demonstrated between the capsulate type b *H influenzae* and a non-capsulated *H influenzae* All the antigens showed 100% cross reaction, except antigens Nos 9 and 23, which cross reacted to a minor degree (25%) (Table 1)

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A



B

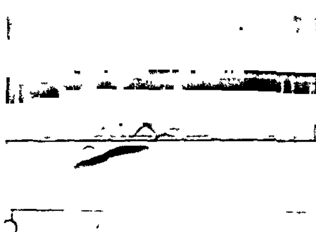


Fig 3 Comparison of antigens from *Haemophilus parainfluenzae* with *Haemophilus influenzae* antigens (H inf Ag) using rabbit anti *H influenzae* antiserum (H inf Ab)

A *Crossed immunoelectrophoresis of H parainfluenzae* antigens against H inf Ab Saline in the intermediate gel 23 distinct precipitates are visible

B Crossed line immunoelectrophoresis of H inf Ag against H inf Ab with *H parainfluenzae* antigens in the intermediate gel (absorption of antibodies *in situ*) 15 of the precipitates of the reference pattern have not been absorbed and 14 of these could be identified The remainder of the precipitates of the reference pattern have been removed or elevated and corresponding straight precipitate lines showing reaction of identity with the elevated reference precipitates are seen in the gel Control reference pattern of H inf -Ag against H inf Ab is seen in Fig 2A Technical data as Fig 2

Ag is insufficient for raising antibodies against surface polysaccharides

In accordance with previous *investigations (13, 19, 23)*, the present study demonstrates extensive cross-reactions between strains of the same species less extensive cross-reactions between different species of the same genus and few cross reactions between different genera (Table 1) Also in accordance with previous results we found that some cross-reactive antigens – notably Nos 4 and 16 – were present in many Gram negative bacteria (13 19, 23)

Anti-capsular antibodies have been shown to be associated with *immunity to infections with type b H influenzae* in humans also (7, 8) Such antibodies may be induced by related polysaccharides from other encapsulated bacteria (31) However, recent studies have shown that also antibodies directed against non capsular *H influenzae* antigens have a part to play in protective immunity in *H influenzae type b infections, at least in animal experiments (10* 28, 29) The importance of the antigens of *H influenzae* which cross react with many other Gram-negative bacteria with regard to protective immunity in humans is, however not yet known It has been shown that antibodies against *H influenzae* antigen No 4 are present in most human sera and in γ globulin preparations (12, 15, 20), and the present study shows that they are also present in rabbit sera before immunization with H inf -Ag *Further studies are needed to ascertain whether* these antibodies may participate in immunity to capsulate as well as non-capsulate *H influenzae* infections

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for providing *Haemophilus influenzae* type b capsular polysaccharide vaccine

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Acta path microbiol scand Sect. B 87 345–351, 1979

PRECIPITATING ANTIBODIES AGAINST *HAEMOPHILUS INFLUENZAE* AND *STAPHYLOCOCCUS AUREUS* IN SPUTUM AND SERUM FROM PATIENTS WITH CYSTIC FIBROSIS

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Schiøtz P O & Høiby N Precipitating antibodies against *Haemophilus influenzae* and *Staphylococcus aureus* in sputum and serum from patients with cystic fibrosis Acta path microbiol scand Sect B 87 345–351 1979

Serum and sputum sol phase from 23 patients with cystic fibrosis (CF) were examined for occurrence and titres of precipitins against *Haemophilus influenzae* and *Staphylococcus aureus* by means of crossed immunoelectrophoresis with intermediate gel The patients had from four to nine *H influenzae* precipitins in serum and in most cases fewer precipitins in sputum but, on an average there was no difference between the titres of the antibodies in serum and sputum Most of the antibodies were cross reactive with other species notably those of the *Haemophilus* genus *S aureus* precipitins were generally found in higher numbers in serum than in sputum but on an average the titre of the precipitins in sputum was higher than in serum Three of the precipitins were detectable only in sputum and not in serum and one of these is a *S aureus* specific precipitin Most of the antibodies were cross reactive with other species and these antibodies were often present in sputum in much higher titres than in the corresponding sera Antibodies against teichoic acid of the *S aureus* cell wall could not be demonstrated in sputum while they were present in 22 sera The possible role of the local pulmonary humoral immune response in protective immunity and in the pathology of the lung disease in CF is discussed

Key words Cystic fibrosis antibodies *S aureus* *H influenzae* local immunity sputum

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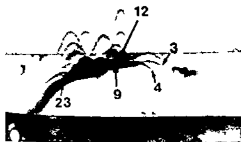
Patients with cystic fibrosis (CF) suffer from chronic and recurrent bacterial lung infections (5 8 9) The three most important pathogens in CF are *Pseudomonas aeruginosa* *Staphylococcus aureus* and *Haemophilus influenzae* (5) and the patients are characterized by a pronounced and heterogeneous humoral immune response against these bacteria (4 7) With regard to *P aeruginosa* the specific anti *P aeruginosa* antibodies circulate in the patients sera but do not promote elimination of *P aeruginosa* from the lungs although the humoral immune response possibly keeps the infection localized to the respiratory tract (6)

The local pulmonary humoral immune response against *P aeruginosa* has been examined previously, and it was found that sputum from chronically infected patients generally contained antibodies against *P aeruginosa* with the same specificities as the antibodies demonstrated in serum but usually with lower titres (10) However, some of these antibodies were directed against *P aeruginosa*

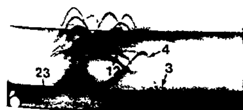
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A



B



C



Fig 1 Crossed immunoelectrophoresis with intermediate gel of 2 µl *Haemophilus influenzae* antigen (H inf Ag) in the well against rabbit antibodies against H inf Ag in the second dimension reference gel (20 µl/cm²) Technical data 1 dimension electrophoresis anode to the right 2 dimension electrophoresis anode at the top Staining Coomassie Brilliant Blue

A Control plate with saline in the intermediate gel Five precipitates are indicated by arrows

B The intermed

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with 1A

C The intermediate gel contains sputum sol phase from the same patient as in Fig 1B

Four precipitates could be revealed in this sputum and are indicated with arrows and numbers Precipitates nos 3 and 4 are seen to have inward feet (lowest titre) Compare with 1A and B

Fig 1 A, B & C shows an example of corresponding immunoplates for investigation of *H influenzae* precipitins, and in Fig 2 A, B & C the same is demonstrated with regard to *S aureus* precipitins Free antigens from *H influenzae* or *S aureus* were not demonstrated either in serum sputum sol phase or in saliva

None of the six saliva specimens from the patients contained any precipitins against *H influenzae* or *S aureus*

H influenzae *Precipitins*

Precipitins against 16 different *H influenzae* antigens were demonstrated in serum and 9 of these were also present in sputum (Table 2) The precipitins demonstrated in sputum represented specificities which could also be identified in serum, but in addition precipitins of other specificities were present in serum from 20 patients There was one exception to this precipitin no 21 was found in 14 of the sputa and only in two of the corresponding sera

There was positive correlation between the number of precipitins in serum and sputum ($R = 0.54$, $p < 0.02$) but there was no significant difference between the mean titre of the precipitins in sputum and serum (Table 3) The serum titres were generally higher than or equal to, those found in sputum except for precipitin no 21 which was present in the highest titre (7) in all the 14 sputa in which it was demonstrated

Four *H influenzae* antigens (nos 4, 7, 10 and 16) have been shown to cross react with *P aeruginosa* antigens (12) Precipitin no 4 was found in serum from all

to occurrence or titre of this precipitin either in serum or in sputum None of the 23 patients had antibodies against antigen nos 7 or 16 in serum, and only one patient had antibodies against antigen no 10 in serum There was no correlation between the number of *H influenzae* precipitins in serum and the number of *P aeruginosa* precipitins in serum

S aureus *Precipitins*

Precipitins against 13 different *S aureus* antigens were demonstrated in serum Seven of these were also present in sputum, which furthermore contained three different precipitins that were specific

antigens (notably *P aeruginosa* antigen no 10) which cross react with many other Gram negative bacteria

These findings caused us to investigate the local humoral immune response in the respiratory tract of CF patients against the other common pathogens *S aureus* and *H influenzae* to see whether the local pulmonary humoral immune response follows the same pattern as described for *P aeruginosa* and to investigate the occurrence of cross reacting anti bodies

PATIENTS AND METHODS

Patients

Twenty three CF patients were included in the study (16 males and 7 females mean age 14 years range 10–26 years) All patients had a typical history of CF and markedly elevated sweat electrolytes in repeated tests (3) These patients are observed in the CF Clinic of Rigshospitalet Copenhagen as described previously (4 5)

Fourteen of the patients had chronic pulmonary infections with mucoid strains of *P aeruginosa* (CF + P) and multiple precipitins against this bacterium in serum at the time of investigation (5 6) The mean number of *P aeruginosa* precipitins was 21 range 4–45 Nine patients had no *P aeruginosa* lung infection (CF–P) and 0–1 *P aeruginosa* precipitins in serum

With regard to pulmonary infections with *H influenzae* and *S aureus* all 23 CF patients had experienced several pulmonary infections with these bacteria previously

Sputum

A 3 h sample of sputum was collected at 4 °C from each patient between 8 a m and 11 a m as described previously (10) Each sample was subjected to bacteriolo gical examination and the origin of the specimens from the lower respiratory tract was confirmed by study of the epithelial cells present (5) Sol phase of sputum (1) was obtained by ultracentrifugation at 120 000 × *g* (maximum value) at 4 °C for 4 h and the samples were stored in small aliquots at −80 °C (10) One sample per patient was investigated

Saliva

One hour unstimulated mixed saliva samples (1–3 ml) were obtained from six of the patients included in the study The saliva samples were centrifuged and stored as described for sputum

Serum

Serum was obtained from each patient on the day when sputum was collected and was stored at −30 °C with NaN_3 added (1‰)

Crossed Immunoelectrophoresis

The occurrence specificities and titres of precipitating antibodies against *H influenzae* and *S aureus* were investigated by means of crossed immunoelectrophoresis with intermediate gel by microtechnique using a polyvalent *H influenzae* reference antigen/antibody and a polyvalent *S aureus* reference antigen/antibody as described previously (12 13)

The concentration of serum sputum sol phase or saliva in the intermediate gel was 20 µl/cm²

The identity and titre of human precipitins in serum sputum sol phase or saliva were determined in relation to the reference pattern according to previously described methods (10) and quantification was performed by comparing the area including human precipitins with the area of corresponding rabbit precipitins in six standard plates The standard plates were run exactly as described above but instead of human fluids in the intermediate gel the standard plates included rabbit antiserum in the intermediate gel as described previously (10) The following titre classes were employed in the present study both for the *H influenzae* reference system and the *S aureus* reference system

0 µl/cm² < titre 1 ≦ 1 µl/cm² < titre 2 ≦ 5µl/cm² < titre 3 ≦ 10µl/cm² < titre 4 ≦ 20 µl/cm² < titre 5 ≦ 40 µl/cm² < titre 6 ≦ 80 µl/cm² ≦ titre 7 A control plate with saline in the intermediate gel was included every day In some instances additional plates with lower concentrations of sputum in the intermediate gel were run to facilitate indentification of the precipitins

Statistical Methods

The Mann Whitney test Wilcoxon s test for pair differences and Spearman s correlation coefficient R (2) were employed Level of significance 5 % (double tailed test)

RESULTS

It was shown that all the patients had precipitins in serum as well as in sputum against both *H influenzae* and *S aureus* The number of precipitins in serum was on an average higher than the number of precipitins in sputum ($p < 0.01$) against both *H influenzae* and *S aureus* (Table I)

TABLE I *Mean* ($\bar{x}$) *and Range (r) of Number of* Haemophilus influenzae *Precipitins and* Staphylococcus aureus *Precipitins in Serum and Sputum of 23 Patients with Cystic Fibrosis*

Precipitins against	Mean and range of number of precipitins in serum	in sputum
Haemophilus influenzae	$\bar{x}$ 5 6 r 4–9	$\bar{x}$ 3 7 r 1–7
Staphylococcus aureus	$\bar{x}$ 5 1 r 2–8	$\bar{x}$ 3 7 r 1–8

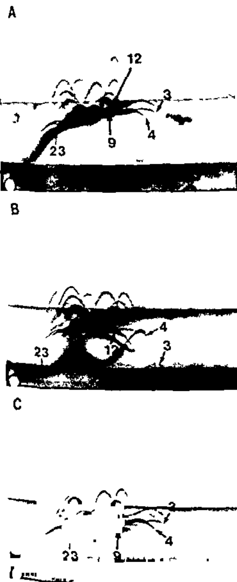


Fig 1 Crossed immunoelectrophoresis with intermediate gel of 2 µl *Haemophilus influenzae* antigen (H inf Ag) in the well against rabbit antibodies against H inf Ag in the second dimension reference gel (20 µl/cm2) Technical data 1 dimension electrophoresis anode to the right, 2 dimension electrophores
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C The intermediate gel contains sputum sol phase from the same patient as in Fig 1B
Four precipitates could be revealed in this sputum and are indicated with arrows and numbers Precipitates nos 3 and 4 are seen to have inward feet (lowest titre) Compare with 1A and B

Fig 1 A, B & C shows an example of corresponding immunoplates for investigation of *H influenzae* precipitins, and in Fig 2 A, B & C the same is demonstrated with regard to *S aureus* precipitins Free antigens from *H influenzae* or *S aureus* were not demonstrated either in serum, sputum sol phase or in saliva

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H influenzae *Precipitins*

Precipitins against 16 different *H influenzae* antigens were demonstrated in serum and 9 of these were also present in sputum (Table 2) The precipitins demonstrated in sputum represented specificities which could also be identified in serum, but in addition precipitins of other specificities were present in serum from 20 patients There was one exception to this, precipitin no 21 was found in 14 of the sputa and only in two of the corresponding sera

There was positive correlation between the number of precipitins in serum and sputum ($R = 0.54$, $p < 0.02$) but there was no significant difference between the mean titre of the precipitins in sputum and serum (Table 3) The serum titres were generally higher than, or equal to, those found in sputum except for precipitin no 21 which was present in the highest titre (7) in all the 14 sputa in which it was demonstrated

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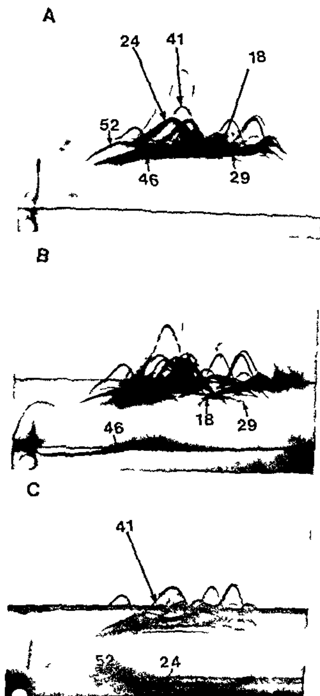


Fig 2 Crossed immunoelectrophoresis with intermediate gel of 5 µl *Staphylococcus aureus* antigen (*S aureus* Ag) in the well against rabbit antibodies against *S aureus* Ag in the second dimension reference gel (20 µl/cm²) Technical data as Fig 1
A Control plate with saline in the intermediate gel Six precipitates are indicated by arrows
B The intermediate gel contains serum from one of the patients Three precipitates could be revealed in this serum and are indicated with arrows and numbers Compare with 2A

and numbers
Precipitins nos 24 and 52 are seen faintly and confluent just cathodic to the intermediate gel

TABLE 2 *Prevalence of the Different* Haemophilus influenzae *Precipitins in Serum and Sputum of Patients with Cystic Fibrosis*

Precipitin specificities[a)]	No of patients with precipitins in serum	in sputum
2	4	1
3	9	4
4	23	15
8	1	0
9	23	20
10	1	0
12	1	0
17	2	0
20	2	0
21	2	14
22A	2	0
23	23	16
25	1	0
28	9	4
29	16	7
33	2	1

[a)] Enumeration according to previously published H inf Ag/H inf Ab reference system (12)

(21 37 41) (Table 4) and two precipitins (24 52) that in some patients were present only in sputum (Table 4) The other precipitin specificities demonstrated in sputum were also found in the corresponding serum Several precipitins were present only in serum (Table 4) particularly no 18 (directed against teichoic acid) which was present in all the serum and in none of the sputum samples (Table 4) There was positive correlation between the number

TABLE 3 *Distribution into Titre Classes of* Haemophilus influenzae *Precipitins in Serum and Sputum of 23 Cystic Fibrosis Patients*

Titre Class	Number of precipitins in serum	in sputum
7	1	14
6	0	0
5	6	0
4	5	0
3	9	1
2	30	4
1	18	62
Geometrical mean of titres	2 3	2 1 n s

Precipitins in serum are only included in this table if precipitins of the same specificities are found in sputum from the same patient

TABLE 4 *Prevalence of the Different* Staphylococcus *aureus Precipitins in Serum and Sputum of Patients with Cystic Fibrosis*

Precipitin specificities*)	No of patients with precipitins in serum	in sputum
6	22	17
10	3	0
12	7	1
13	1	0
14	4	0
15	8	1
18	22	0
21	0	4
24	8	23
26	1	0
29	9	0
37	0	5
41	0	11
46	23	5
47	2	1
52	11	15

*) Enumeration according to previously published *S aureus* Ag/*S aureus* Ab reference system (13)

of precipitins in serum and sputum (R 0 76 p < 0 001)

The titres of the *S aureus* precipitins are shown in Table 5 from which it appears that the mean titres of the precipitins were significantly higher in sputum that in serum (p < 0 001)

S aureus antigen no 24 has been shown to cross react with a *P aeruginosa* antigen (13) The

TABLE 5 *Distribution into Titre Classes of* Staphylococcus aureus *Precipitins in Serum and Sputum of 23 Cystic Fibrosis Patients*

Titre Class	Number of precipitins in serum	in sputum
7	0	16
6	0	1
5	2	4
4	3	6
3	3	26
2	3	10
1	26	21
Geometrical mean of titres	1 7	3 3 p<0 001

Precipitins in serum are only included in this table if precipitins of the same specificities are found in sputum from the same patients

occurrence and titre of this precipitin did not differ significantly between the CF + P patients and the CF P patients either in serum or in sputum There was positive correlation between the number of *S aureus* precipitins in serum and the number of *P aeruginosa* precipitins in serum (R − 0 57 p < 0 01)

There was also positive correlation between the number of *S aureus* precipitins in sputum and the number of *H influenzae* precipitins in sputum (R − 0 68 p < 0 005) while there was no correlation with regard to number of these precipitins in serum

DISCUSSION

H influenzae *Precipitins*

In general the local humoral immune response to *H influenzae* though less pronounced resembles that of *P aeruginosa* (10) with fewer precipitins and lower titres of these in sputum than in serum This fact is also reflected in the low correlation coefficient (R − 0 54) between the number of precipitins in serum and sputum

It was only against the cross reactive antigen no 21 (12) that a high titred local humoral immune response was demonstrated

Ninety-eight per cent of the precipitins in sputum and 98% of the precipitins in serum were directed against *H influenzae* antigens cross reactive with other *Haemophilus* species (12) Twenty two per [illegible]

[illegible] other bacterial genera (12) Therefore only 2% of the sputum and serum precipitins indicate a specific antibody response against *H influenzae* in the CF patients although the cross reactive antibodies could also be induced by *H influenzae*

In spite of the presence of the cross reactive antibodies in serum no correlation was found between the number of precipitins against *H influenzae* and either *P aeruginosa* or *S aureus* There was however correlation between the number of *H influenzae* precipitins and the number of *S aureus* in sputum This may be due to the fact that such patients frequently harbour these two bacterial species together in the lungs (5) whereas cross reactive antibodies could not be the reason (12 13)

Further characterization of some of the precipitins is possible Precipitin no 21 is directed against a cross reactive surface antigen (12) Precipitin no 23 is directed against a polysaccharide-containing heat stable antigen possibly related to *H influenzae*

lipopolysaccharide and precipitin no 9 against a surface antigen neither of which are cross reactive (12)

Antibodies against the capsule of *H influenzae* type b were not demonstrated although *H influenzae* type b capsular polysaccharide is present in the antigen preparation employed (12) If present in CF serum and sputum the concentration of these antibodies must be low or possibly non precipitating

S aureus *Precipitins*

On an average the titre of the local antibody response against *S aureus* antigens was higher than the titre of the systemic antibody response and the local antibody response seems to be relatively more pronounced as compared to that against *H influenzae* and *P aeruginosa* (10) Furthermore three precipitins were completely specific for sputum and two (nos 24 and 52) were present in higher titres in sputum than in serum thus indicating a local pulmonary antibody response

The reason for the pronounced local antibody response against *S aureus* is unknown but it might be related to the well known tendency of *S aureus* to cause localized infections (abscesses) possibly due to the production of coagulase and protein A

Sixty per cent of the precipitins in sputum and 54% of the precipitins in serum were directed against *S aureus* antigens cross reactive with *S epidermidis* (13) Forty per cent of the precipitins in sputum (nos 24 41 47 and 52) and 31% of the precipitins in serum (nos 13 24 47 and 52) were directed against *S aureus* antigens cross reactive with other bacterial genera (13) It is therefore only *40% of the sputum precipitins and 46% of the* serum precipitins which indicate a specific antibody response against *S aureus* in CF patients although the cross reactive antibodies could also be induced by *S aureus* The correlation between the number of *S aureus* and *P aeruginosa* precipitins in serum cannot be accounted for by cross reactive antibodies since only one cross reactive antigen exists between these species However *S aureus* and *P aeruginosa* alone or in combination are the prevalent bacteria in CF (5) and this may be the reason for the correlation between the antibody response in serum against these bacteria

Further characterization of some of the precipitins is possible Precipitin no 18 is directed against teichoic acid while precipitin no 46 is directed against a heat stable polysaccharide-containing antigen (13) Antibodies against both these antigens are cross reactive and are thus not specific for *S aureus* (13)

In general cross reactive antibodies which predominated in the immune response did not see provide protective immunity against *S aureus* *influenzae* pulmonary infections in CF A sp local pulmonary immune response was also de strated against both these bacteria but its ro local protective immunity requires further s The presence of *S aureus* and *H influe* antibodies in the lungs of CF patients indic however that chronic lung infection in CF pat with these bacteria may induce immune com *mediated* tissue damage since it has been sh previously that chronic *P aeruginosa* lung infe in CF is an immune complex disease (10 11)

This work was supported by the *Danish Me Research Council* (project no 512 5267 6623 8115) the *National Association for the Fight ag Tuberculosis* and the *National Dan sh Associat on ag Cystic Fibrosis*

We thank *Anni Bethien* and *Ellinor Ward Peterse* skilful technical assistance

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Acta path microbiol scand Sect. B 87 353-362 1979

EXPERIMENTAL ENDOCARDITIS IN RABBITS

3 *Significance of the Proteolytic Capacity of the Infecting Strains of* Streptococcus Faecalis

ERNÖ GUTSCHIK SUSANNE MØLLER and NILS CHRISTENSEN

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Gutschik E Møller S & Christensen N Experimental endocarditis in rabbits 3 Significance of the proteolytic capacity of the infecting strains of *Streptococcus faecalis* Acta path microbiol scand Sect B 87 353-362 1979

Insertion of a polyethylene catheter into the left ventricle of the heart was used for regular establishment of sterile endocarditis and bacterial endocarditis was established by injection of approximately 10[8] *Streptococcus faecalis* into the blood stream at the same time as removal of the catheter which had been in place for 3 days 100 out of 102 rabbits died spontaneously of bacterial endocarditis Evidence is produced that the host parasite interaction is influenced by the proteolytic property of *S faecalis* in this experimental model Two distinct types of clinical course are described 1) A predominantly acute and damaging illness characterized by a high level of bacteraemia small amounts of soft, friable vegetations in the left side of the heart, high frequency of kidney infarcts and shorter survival time in rabbits infected with proteolytic strains 2) A relatively subacute illness characterized by a lower level of bacteraemia large hard non friable vegetations on the aortic valves less pronounced destructive changes in the substance of valve leaflets relatively lower frequency of kidney infarcts and longer survival time in rabbits infected with non proteolytic strains The results suggest that proteolytic strains of *S faecalis* cause partial dissolution of the vegetations resulting in a more severe clinical picture

Key words Experimental endocarditis rabbits *Streptococcus faecalis* proteolytic capacity

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Received 11 i 79 Accepted 1 vi 79

The spontaneous course of experimental *Streptococcus faecalis* endocarditis in rabbits has been described in a previous report (8) using a model originally developed by *Garrison & Freedman* (5) This model has been found to be simple and reliable for investigation of the pathogenesis of sterile and infective endocarditis (2 3 4 5 11) The main modification used in our laboratory is the improved technique of catheterization (7) and the removal of the catheter after insertion for 3 days at the same time as inoculation of the rabbit with *S faecalis* Growth of the bacteria took place in the preformed vegetations resulting in high density of bacteria inside the vegetations (8) While investigating this improved rabbit model of experimental endocarditis it was observed that proteolytic (gelatin liquefying) and non proteolytic (non gelatin liquefying) strains of *S faecalis* showed different pathogenic effects Since the vegetations are composed mainly of fibrin (4) the spontaneous course of infection could be influenced by the proteolytic (fibrinolytic) capacity of the infecting strains

In order to substantiate this observation 102 rabbits were infected experimentally with 10 strains of *S faecalis* with varying gelatin liquefying ability

TABLE 1 *Design of Experiments*

Rabbit groups	Subgroups		Biochemical pattern of infecting strains		Taxonomic designation of infecting strains
	Number of rabbits	Strain no	Gelatin liquefaction	β haemolysis	
I	10 10 10	1 2 3	+	+	*S faecalis* *Subsp zymogenes*
II	10 10	4 5	+	–	*S faecalis* *Subsp liquefaciens*
III	10 10	6 7	–	+	*S faecalis* *Subsp zymogenes*
IV	10 10 10	8 9 10	–	–	*S faecalis* *Subsp faecalis*

negative blood cultures for 28 days after inoculation with bacteria These two animals were inoculated with strains no 4 (gelatin liquefying non β haemolytic) and no 7 (non gelatin liquefying β haemolytic) respectively No sign of infective endocarditis was found on autopsy but whitish fibrous changes in the bottom of the left ventricle indicated the previous catheterization

Survival Time

Rabbits inoculated with proteolytic strains of *S faecalis* were characterized by significantly shorter survival times than those inoculated with non proteolytic strains This is demonstrated by the significant difference in the average survival time between rabbits in groups I + II and in groups III + IV (Table 2 Fig 1) No significant difference

TABLE 2 *Clinical Data for Rabbits Succumbed Spontaneously to Left sided* Streptococcus faecalis Endocarditis

		Biochemical pattern of infecting strains				Standard deviation (%)
		Gelatin liquefying strains		Non gelatin liquefying strains		
		β-haem Gr I	Non β haem Gr II	β haem Gr III	Non β-haem Gr IV	
rvival time of bits (hours)	$\bar{x}$ n	119 7 30	94 4 20	233 9 20	170 6 30	57
eight of etations (mg)	$\bar{x}$ n	88 7 24	92 3 17	266 1 16	299 9 24	50
eight of leen (g)	$\bar{x}$ n	2 11 30	2 08 20	3 75 20	2 99 30	44
eight of (g)	$\bar{x}$ n	8 26 30	8 89 20	9 59 20	9 23 30	14
in vegetations) °CFU/g vegetation)	$\bar{x}$ n	6 21 24	8 43 17	9 02 16	5 45 24	186

~ geometric mean n = number of rabbits CFU = colony forming units

MATERIALS AND METHODS

The experimental animals were random bred male albino rabbits (Ssc CPH) from Statens Seruminstitut The average weight was 2976 g (SD 125 g) and the average age 203 6 days (SD 36 4 days)

Pretreatment for producing left sided sterile endocarditis The rabbits were anaesthetized intravenously with mebumal natrium The average dose at the first operation was 23 8 mg/kg (SD 2 7) and at the second operation 22 8 mg/kg (SD 2 2) A polyethylene catheter was inserted into the left ventricle of the heart using the technique described previously (7) and left *in situ* for 3 days

Infection of pretreated animals After 3 days with the indwelling catheter a second operation was performed to remove the catheter and 1 ml of a bacterial suspension of *S faecalis* was injected simultaneously into a marginal ear vein The suspension in saline was prepared from an overnight culture grown on 5% blood agar This suspension contained approximately 10^8 colony forming units (CFU) per ml (mean $1\ 37 \times 10^8$ range $6\ 13 \times 10^7 - 2\ 19 \times 10^8$)

Infecting organism Ten strains of *S faecalis* with varying ability to liquefy gelatin and to produce β haemolysis were used (Table 1) These strains were obtained from clinical material and have been characterized previously (6) together with the media and the tests used for cultivation and identification

Bacteria recovered from blood vegetations and different organs from the experimental animals were examined for haemolytic activity on 5% horse blood agar plates studied on Gram stained slides and tested for tellurit resistance and gelatin liquefaction

Quantitative blood culture Blood specimens were taken every day during the infection period until spontaneous death or the predetermined time of sacrifice (28 days) For cultivation 0 5 ml blood was taken from an ear vein into a syringe previously filled with 0 5 ml saline containing 10 U heparin (Leo Laboratories Copenhagen) per ml The whole amount was streaked immediately onto three 5% blood agar plates (diameter 13 5 cm) When a larger number of CFU was expected a further dilution of 1 20 was made in 0 9% saline and 1 ml of this dilution was streaked onto blood agar plates The plates were incubated at 35° C and the number of colonies was counted 48 hours later Besides bacterial cultivation 2–4 ml blood was drawn daily from each rabbit for other purposes

Autopsy All cages were inspected at least every 6 hours day and night so that in case of spontaneous death animals could be removed to a refrigerator and kept there until autopsy could be performed The survival time of rabbits was calculated as the interval in hours between bacterial challenge and spontaneous death If the exact time of spontaneous death was not known the mean time between the last time the rabbit was observed alive and the time of death was used The heart and spleen were weighed and accumulated fluid from the pleural spaces and peritoneal cavity was collected and quantified The vegetations from the left side of the heart were removed aseptically and homogenized in glass tissue grinders Quantitative culture was carried out as described earlier (8) The number of organisms recovered was expressed as CFU/g wet tissue

Semiquantitative culture was performed from the peritoneal cavity liver spleen kidneys mesenteric glands and pleural cavity as described previously (8)

The hearts of two rabbits in each subgroup (except one heart from subgroup 4) were used exclusively for histological examination of sections from the aorta at the level of the orifice of a anonyma above the leaflets from the aorta ostium from underneath the ostium from the septum and papillary muscles and from the myocardium at the bottom of the left ventricle

The gross structure of the liver spleen mesenteric glands kidneys and suprarenal glands was examined Where pathological processes were observed or suspected histological examination was also carried out

Blood urea nitrogen was evaluated according to Levine et al (9)

Statistical methods Evaluation of the variation between subgroups within the groups and between groups was made by means of a one way nested analysis of variance The analysis was made for each of the variables survival time weight of heart weight of spleen weight of vegetations and CFU/g vegetation after a logarithmic transformation required to obta normal distributions The bacterial counts from t blood were also transformed logarithmically and tw way analysis of variance was carried out for estimatio of the variation between animals and between days counting The assumption of additivity which corr sponds to parallelism of the curves relating to log CF and day was tested These analyses were carried out f each subgroup and also for each group For each day observation the deviation of the observation from that the first day was calculated The difference of the mea deviation from zero was tested by means of t test and th same test was used for pairwise comparisons between th groups It should be noted that the tests carried out fo the different days were not independent

Relative frequencies were compared by Fisher s Exac Test and correlation between variables was tested b Spearman s Rank Correlation Test

General plan of the study A total of 102 rabbits with sterile endocarditis were inoculated with 10 different strains of *S faecalis* Quantitative blood cultures were carried out in all rabbits until they died from their infection except in the case of two rabbits sacrificed 28 days after inoculation because of spontaneous recovery These two rabbits are excluded from the statistical analysis In order to evaluate differences in the clinical data between rabbits inoculated with *S faecalis* displaying different biochemical patterns the animals were divided into four experimental groups and 10 subgroups (Table 1)

RESULTS

Mortality

Death after infection occurred in 100 out of 102 rabbits (98 %) within 28 days Two rabbits showed

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TABLE 3 *Two sided Variance Analysis (Animals × days) of Colony Forming Units in Blood Specimens Taken from Rabbits with Experimental* Streptococcus faecalis *Endocarditis*

Rabbits: Groups	Rabbits: Subgroups	s^2	f	Bartlett s test for common variance (%)	F test for parallelism between subgroups (%)
I	1	0 3822	22		
	2	0 4975	41	70–80	
	3	0 4073	28		
Within subgroups		0 4419	91		
Between subgroups		0 6784	17		10
II	4	0 5908	26	5	
	5	0 2481	19		
Within subgroups		0 4461	45		
Between subgroups		0 2361	5		70–90
III	6	0 5462	76	50–60	
	7	0 6307	70		
Within subgroups		0 5867	146		
Between subgroups		0 6793	18		30
IV	8	0 2913	51		
	9	0 4562	56	10–20	
	10	0 2728	44		
Within subgroups		0 3471	151	10–20	
Between subgroups		1 1677	21		<0 05

s^2 variance f degrees of freedom

rabbits but these were not clearly related to proteolytic and non proteolytic strains of *S faecalis* However in rabbits inoculated with non proteolytic strains significant correlation was found between the heart weight and the weight of vegetations (Spearman s Rank Correlation Test P <0 01) Such a correlation could not be demonstrated in rabbits infected with proteolytic strains There were no differences correlated with the β haemolytic ability of strains (Table 2) An analysis discriminating between heart weight ≧10 g and <10 g showed a significantly higher frequency of hypertrophic hearts (≧10 g) in groups of rabbits inoculated with non proteolytic strains Thus 12 rabbits (5 + 7) in groups I + II had heart weight ≧10 g as compared to 26 rabbits (12 + 14) in groups III + IV (P < 0 001 Fisher s Exact Test)



Fig 3 Bacteraemia in 100 rabbits with left sided Streptococcus faecalis endocarditis registered by daily quantitative blood culture until spontaneous death
D Mean value of increase in log CFU/ml from day 1 for surviving rabbits
O—O Group I (rabbits infected with proteolytic β-haemolytic strains)
Δ—Δ Group II (rabbits infected with proteolytic non β h…
●—●
▲—▲

was found between groups of rabbits inoculated with strains differing in their β-haemolytic reaction (Table 2, Fig 1)

Appearance, Weight and Bacteriology of the Vegetations

In all rabbits, vegetations were found on the aortic valves, in the left ventricle, on the aortic wall a few mm above the leaflets and occasionally around the orifice of a anonyma Vegetations on the atrioventricular valves in the left ventricle were found in 12 out of 50 rabbits inoculated with proteolytic strains, but in 21 of 50 rabbits inoculated with non-proteolytic strains This difference is not significant (P = 0 088)

Two distinct types of vegetations were observed The vegetations infected with proteolytic strains were greyish-white, soft and friable, whereas those infected with non-proteolytic strains were off-white, hard and non-friable The extension of the soft, friable vegetations was moderate and never obstructed the orifice of the aorta, even though all three

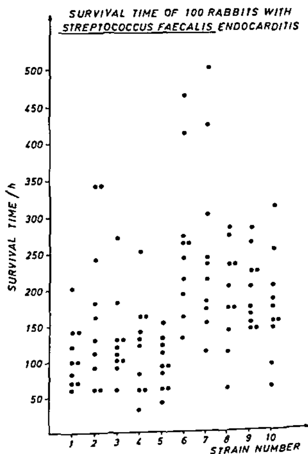


Fig 1 Ten different strains were used for inoculation of subgroups of 10 rabbits Each dot represents one rabbit Strains 1–5 (groups I + II) rabbits inoculated with proteolytic strains Strains 6–10 (groups III + IV) rabbits inoculated with non proteolytic strains

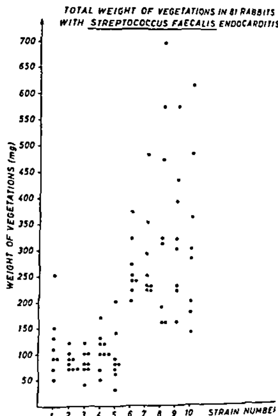


Fig 2 Design of experiment as in Fig 1 Data reco for 81 animals only since 19 hearts were used exclusi for histological examination Strains 1–5 proteol; Strains 6–10 non proteolytic

aortic valve leaflets were always found to involved Leaflet shape was always recogniz (Figs 4A and 5A) The hard, off-white, non-fri vegetations were large and usually obstructed orifice of aorta, even causing some distension of aortic wall Valve leaflets were hardly recogniz (Figs 6A and 7A)

The weight of vegetations infected with protec tic strains was significantly lower than that vegetations infected with non-proteolytic stra (Table 2) Although a rather high variance v observed inside the subgroups, the two types vegetations had a clearly different distribut (Fig 2)

Bacterial density was high in both types vegetations The average number of CFU var from 0 82 × 10^{10} to 6 34 × 10^{11} per g vegetatic Some variations were observed between subgrou of rabbits inoculated with individual strains of *faecalis*, but there were no significant differen between the main experimental groups

Weight of Heart

With regard to heart weight, one-sided varian analysis revealed differences between the groups

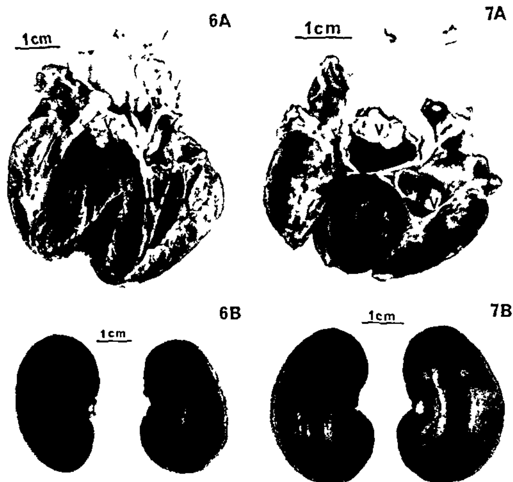


Fig 6A Heart of a rabbit infected with non proteolytic β haemolytic strain (no 7) The survival time was 237 hours Weight of vegetations is not recorded because of histological examination Note the large vegetation obstructing the orifice of aorta The shapes of the aortic valve leaflets are not recognizable

Fig 6B Kidneys belonging to the same rabbit are without infarcts

Fig 7A Heart of a rabbit infected with non proteolytic β-haemolytic strain (no 6) The survival time was 407 hours and the weight of vegetations 271 7 mg The appearance of vegetations resembled that shown in Fig 6A

Fig 7B Kidneys belonging to the same rabbit are without infarcts

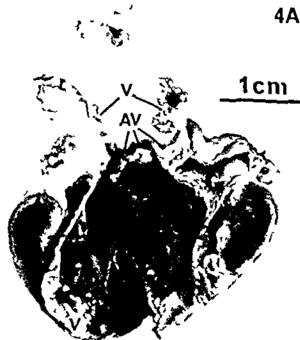
Symbols AV aortic valves with vegetations V = vegetations I = infarcts

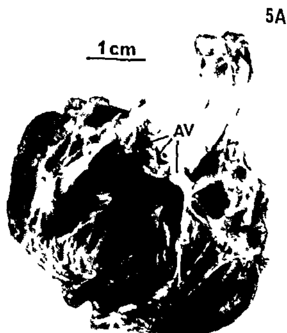
the underlying tissues *i e* the substance of valve leaflets aortic wall or myocardium

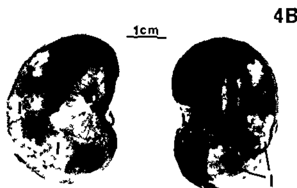
The most marked differences were observed on the aortic valves Here the aggregates of proteolytic bacteria tended to be closer to the luminal surface a tendency which was less marked for the non proteolytic bacteria and not observed in non aortic valve vegetations The destructive changes in the substance of aortic valve leaflets were more pronounced for the proteolytic bacteria. Outside the aortic valves distinct differences in the degree of bacterial density localization of the bacterial aggre gates or changes in the underlying tissues showed only insignificant variations independent of the type of infecting bacteria

Quantitative Blood Culture

By cultural analysis of 627 blood specimens from 100 rabbits taken daily during the entire infection period it was found that only 14 (2 23%)







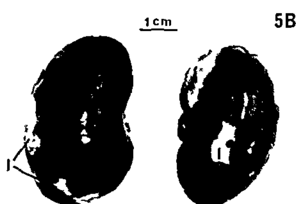


Fig 4A Heart of a rabbit infected with proteolytic β haemolytic strain (no 3) Survival time 125 hours weigh vegetations 71 1 mg Note the moderate amount of vegetations and the easily recognizable shapes of the three ao valve leaflets

Fig 4B Kidneys belonging to the same rabbit have severe infarction

Fig 5A Heart of a rabbit infected with proteolytic β haemolytic strain (no 2) In spite of an unusually long surv time (336 hours) the weight of vegetations was 92 9 mg and the appearance resembled that shown in Fig 4A

Fig 5B Kidneys belonging to the same rabbit have severe infarction

Histopathology of Heart

The purpose of the histological examination was to detect possible morphological differences between vegetations infected with proteolytic and non proteolytic strains of *S faecalis* The systematic examination included observation of the density of bacteria in the fibrin mass, the shape and size of bacterial aggregates in the vegetations, possible variations in the distance between bacterial aggregates and the luminal surface of the vegetations the degree of endothelialization of vegetations and t destructive changes in the underlying tissues

Several common trends were observed for bo types of vegetations Both contained masses bacteria with density always greatest in the veget tions on the aortic valves, while the tendency fo endothelialization was less there The bacteri aggregates occurred as oval or circular rings o festoons, independent of the type of infectin bacteria Finally, bacteria were never observed i

baseline values (before infection) was only observed 1 2 days before death probably because of dehydration

DISCUSSION

S faecalis causes 5–15% of all cases of human infectious endocarditis (10) In spite of this relatively low frequency this fact deserves special attention because the organism commonly causes endocarditis on normal cardiac valves Furthermore the clinical pattern of infection is often distinct from other streptococcal endocarditis mainly due to the more variable clinical course consisting of a chronic indolent form and an acute rapidly progressive from with early disruption of valve leaflets (12)

A previous incidental observation indicating certain differences between cases of experimental rabbit endocarditis depending on the ability of the infecting strains of *S faecalis* to liquefy gelatin has now been confirmed

Although the differences are mostly of a quantitative nature the number of experiments performed and of strains used together with the statistical analysis leaves little doubt that there is a difference and that the difference is correlated with the ability of the strains to liquefy gelatin

The immediately most striking difference is the appearance and extent of the cardiac vegetations Proteolytic strains give soft friable vegetations that are not quite as extensive as the hard non friable vegetations produced by non proteolytic strains The proteolytic strains also produce more severe infections as shown by a shorter survival time a higher degree of bacteraemia and a larger number of kidney infarcts

It has been shown (*Gutschik* unpublished observation) that the protease of the gelatin liquefying strains also has the ability to dissolve fibrin Since fibrin is the major component of the vegetations it might be assumed that the essential difference is due to the fibrinolytic activity exerted by the numerous and dense aggregates of living bacteria inside the vegetations It can easily be imagined that the fibrinolytic activity will soften the vegetations so that they more easily become eroded or torn to pieces by the high pressure of the blood stream thus resulting in a relative diminution in their extent and also in the finding of more free bacteria in the blood and embolism as shown by the frequent kidney infarcts Why the animals die sooner is not immediately obvious unless it is in some way a consequence of the higher degree of bacteraemia. Apparently it has nothing to do with impaired kidney function

Statistical analysis showed some differences between the average weight of spleen and heart in rabbits infected with proteolytic and non proteolytic strains Significant correlations were not found between the weight of spleen and other single parameters used in this study therefore the difference is possibly due to influence of multiple factors during the infection As regards the weight of the heart significant correlation was found with the weight of the vegetations but only in the group of rabbits inoculated with non proteolytic strains This observation indicates that hypertrophy of the heart can develop due to the large vegetations partially obstructing the orifice of aorta There is reason to suppose that heart hypertrophy in rabbits infected with proteolytic strains is merely due to aortic insufficiency as a result of the destructive changes in the substance of aortic valve leaflets

The borderline value for spleen enlargement $\geqq 2$ g due to infection was found suitable since it had been shown earlier that rabbits with sterile endocarditis do not display this increase in weight (7) Similarly borderline value of $\geqq 10$ g for heart hypertrophy was used because rabbits with sterile endocarditis observed for 5 days all had heart weight below that value (7)

Wretlind & Kronevi (13) have reported on the use of protease-deficient mutants of *Pseudomonas aeruginosa* in experimental infection of mice and reached the conclusion that proteolytic activity had little effect on the outcome Considering that the mice in that case suffered from an overwhelming generalized infection the result is not surprising and does not exclude that in localized infections the protease activity may have important consequences

The significance of differences in the proteolytic activity of the infecting strains in cases of human endocarditis is not known at present but it will be an important future task to study this aspect

Investigations on the pathogenesis of endocarditis involve considerations about the disturbing influence of the indwelling catheter and very high inoculum (10^7–10^8 CFU) as used in the rabbit model originally described by *Garrison & Freedman* (5) and *Durack & Beeson* (2) Our results show unequivocally that the presence of the catheter is not necessary for the induction or for the maintenance of the infection It is *unlikely* that the large inoculum which was also used in this study will influence the spontaneous course of endocarditis Immediately after the injection of inoculum the number of organisms in the blood was found to be 10^5–10^6 CFU/ml but it fell rapidly (during 10–15 minutes) to 10^3–$10^?$ CFU/ml (*Gutschik* unpublished observation) The transitory high level bacteraemia will not produce endocarditis in normal i e

specimens did not show growth of bacteria The level of bacteraemia varied from a few bacteria per ml to 10^5/ml blood Particularly high values were observed just prior to spontaneous death The level of bacteramia during the first 2 days of infection was sometimes low a value $< 10^2$ CFU/ml was determined in 8 out of 50 rabbits inoculated with proteolytic strains (16%) and in 17 out of 50 rabbits (34%) inoculated with non proteolytic strains This difference is not significant (P = 0 063)

Comparison of the levels of bacteraemia during the entire period of infection showed differences between individual animals but the progress of bacteraemia appeared to be parallel within sub groups The homogeneity of the variances and the parallelism have been tested for each of the four experimental groups (Table 3) The table shows that the parallelism was acceptable within groups I II and III but not in group IV where one of the subgroups had a constant low level of bacteraemia This fact made further comparison of the groups by means of estimated average curves difficult and therefore the final evaluation was carried out using the results observed on day 1 as reference value For each animal the increase in log CFU/ml was calculated as deviation from the log CFU/ml observed on day 1 The averages of these deviations calculated for the surviving animals are shown in Fig 3 The averages are significantly greater than zero for all days in groups I and II and for days 4–11 in group IV For days 2–8 significantly greater levels of bacteraemia were found for rabbits inoculated with proteolytic strains As can be seen from the figure the differences are systematic the curves for groups I and II lying above those for groups III and IV No similar difference was found as regards the β haemolytic property of the infecting strains since the curves for groups II and IV (non β haemolytic) are lying between the curves for groups I and III (β haemolytic)

Spleen

Statistical analysis shows that in group IV there were significant differences between the average weights of the subgroups However there was no significant difference between groups III and IV Considering groups I + II and III + IV a significantly higher average spleen weight was found for rabbits inoculated with non proteolytic strains (Table 2)

An analysis discriminating between weight ≧ 2 g and < 2 g showed the same trend 28 rabbits (56%) in groups I + II had weight > 2 g compared with 43 rabbits (86%) in groups III + IV (P = 0 0017) Values of < 2 g were not significantly correlated with shorter survival times examined in t[he] subgroups

Splenic emboli with infarction were recognized [in] four out of 100 rabbits

Kidneys

In rabbits infected with proteolytic strai[ns] (groups I + II) 97 out of 100 kidneys had one [or] more infarcts These lesions were found in only 5 of 100 kidneys in rabbits infected with no[n] proteolytic strains (groups III + IV) The differenc[e] is highly significant (P < 0 001 Fisher s Exact Tes[t] see also Figs 4A B 5A B 6A B and 7A B) Th[e] infarcts were anaemic and mainly cortical coverin[g] from 1–2 mm^2 of the surface to almost half of th[e] kidney An analysis discriminating between kidne[y] infarcts ≧ 10 mm^2 and < 10 mm^2 showed that i[n] rabbits infected with proteolytic strains infarcts ≧ 10 mm^2 were found in 64 out of 97 kidneys whil[e] in rabbits infected with non proteolytic strains onl[y] three out of 58 kidneys displayed this characteristic This difference is highly significant (P < 0 001 Fisher s Exact Test)

Other Organs

Out of 100 rabbits typical infarcts were discove red in the lungs of four and in the liver of one rabbit Except for the kidneys focal infectious processes were only seen as small abscesses in five rabbits One rabbit had paralysis of both limbs Pericardial effusion of 2–5 ml usually sterile fluid was found in 19 rabbits while pleural and peritoneal effusion of more than a few ml was found only in a small number of rabbits With a few exceptions semiquantitative cultivation from the peritoneal cavity and the pleural spaces yielded no growth while in nearly all rabbits strong growth of *S faecalis* was observed from liver spleen kidneys and to a lesser extent from mesenteric glands and lungs No definite difference was observed between rabbits infected with proteolytic and non proteolytic strains

Blood Urea in the Course of Infection

Since blood specimens were taken before inocula tion of rabbits with bacteria and thereafter daily until death it was possible to investigate the level of blood urea as a measure of possibly damaging effect of kidney infarcts Three rabbits infected with proteolytic strains and showing very severe infarc tion of the kidneys (several infarcts > 10 mm) were compared with another group of three rabbits infected with non proteolytic strains and showing no infarcts Regression analysis showed no signifi cant differences between the groups as regards blood urea level Significant increase in blood urea from

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OCCURRENCE AND CHARACTERIZATION OF »AVIUM-LIKE« MYCOBACTERIA ISOLATED FROM ANIMALS IN SWEDEN

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Bergman R & Holmberg O Occurrence and characterization of »avium like« mycobacteria isolated from animals in Sweden Acta path microbiol scand Sect B 87 363–369 1979

431 cultures of »avium like« mycobacteria (ALM) were isolated from wild and domestic animals during 1974–76 at the National Veterinary Institute Stockholm Of these 50 isolates from pigs were studied by growth chromogenicity pathogenicity and biochemical tests Furthermore thin layer chromathography was performed and on some isolates serotyping All 50 isolates belonged to Runyon's group III and were pathogenic for chicken none was capable of splitting oleic acid from Tween 80 47 gave tellurite reduction within a period of three days one was arylsulphatase positive after three days and a further four after 14 days The biological and biochemical tests permit assignation of the 50 isolates to the *M avium intracellulare* complex The lipid patterns of the isolates examined were analysed by thin layer chromatography Thirty five of the isolates showed a lipid pattern similar to that of A 2 of the fowl reference strain three belonged to lipid type A 1 and four to A 3 Eight could not be typed Of 22 isolates 14 could be assigned to *M avium* serotypes

Key words Avium like mycobacteria occurrence characterization animals Sweden

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Infections with mycobacteria occur in man and in both domestic and wild animals and constitute a serious public health problem all over the world

Veterinary bacteriologists have traditionally focused their attention on the fight against tuberculosis and study of the various diagnostic aspects In 1939 before the eradication campaign against tuberculosis in animals in Sweden during the forties *Lilleengen* investigated those types of mycobacteria which could be isolated from pigs (9) On examination of 128 positive cases *Mycobacterium bovis* could be isolated in 116 cases *Mycobacterium tuberculosis* in three and *Mycobacterium avium* in nine The precision of this classification was based on growth colonial morphology pigmentation and guinea pig inoculation

Since then the prevalence of *Mycobacterium bovis* in Sweden has decreased and nowadays this species is seldom isolated from domestic animals However the problem with bacteria previously called *Mycobacterium avium* i e isolates which are slowly growing non pigmented and non virulent for guinea pigs still remains but has attracted little attention and has not been studied in this country until recent years (1) A more suitable working designation for such isolates might be »avium like« mycobacteria (ALM)

The aim of this study was to survey the occurrence of ALM in animals in Sweden Since many isolates are obtained from pigs intended for human consumption porcine strains were characterized further

MATERIAL AND METHODS

Strains

Tissue specimens from domestic and wild animals with lesions reminiscent of tuberculosis were obtained at random and examined at the National Veterinary

non pretreated rabbits (3) Furthermore a bacteraemia of 10^5 CFU/ml is not only part of the subsequent course of experimental endocarditis but may also be seen in rare instances in cases of human septicaemia (1)

It is our conclusion that using a technique similar to ours experimental endocarditis can be established in a form that is well suited not only for testing the effect of various therapeutics but also for a closer study of the influence of various bacterial factors on the disease processes as demonstrated in the present study

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Institute during the period 1974–76 The ALM cultures isolated and used for further characterization originated from various organs of 29 pigs (Table 1) representing 21 herds slaughtered at abattoir No 51 in Kalmar during a one year period (1975) These cultures numbered 1–50 are designated »Kalmar« isolates Four reference strains* of *Mycobacterium avium* three (M5 M8, M80) isolated from fowls and one (NCTC 8551) from a pig were included in the investigations

Culture Technique

Samples from tissue specimens were cultivated on Lowenstein Jensen medium with 0 75% glycerin at 37 °C for a period of eight weeks Strains studied by biological and biochemical tests and thin layer chromatography (TLC) were subcultured for 14 days at 37 °C in an atmosphere of 5% carbon dioxide prior to analysis

Growth chromogenicity and Biochemical Tests

The 50 »Kalmar« isolates selected for further characterization were grouped according to the scheme devised by *Runyon* (13) Three biochemical tests for characterization within Runyon s group III were used

1) Capacity to split phenolphthalein from tripotassium phenolphthalein-disulphate after 3 and 14-day reaction periods according to the method described by *Tarshis* (17) the so-called arylsulphatase test The *M intracellulare* strain NCTC 10425 was used as positive control

2) Tellurite reduction within a period of three days by the method of *Kilburn et al* (7) The *M smegmatis* strain E I from Weybridge was used as positive control

3) Ability of isolates to split oleic acid from Tween 80 by *Wayne s* method (20) The NCTC strain 2275 of *M Kansasii* was used as positive control

Virulence Test

Virulence tests were performed by intravenous inoculation of each of the 1–50 porcine »Kalmar« isolates and the *M avium* NCTC strain 8551 into two 10-day-old chickens respectively In preliminary experiments a loopful (approximately 0 01 ml) of organisms harvested from Lowenstein Jensen medium suspended in 0 1 ml 0 15 M saline and injected into the wing vein was found to be a suitable procedure The inoculated animals were observed for eight weeks Autopsy was performed on chickens which died or became moribund during that period The extent of tuberculosis involvement of liver spleen and bone marrow was assessed by macroscopic and histologic examination

Thin layer Chromatography (TLC)

The method used was that of *Marks et al* (10) as modified by *Boughton & Bernstad* (1) From each of the cultures on Lowenstein Jensen medium a loopful of organ ...

overnight the heterogeneous sample was centrifuged at 500 *g* for 10 min The supernatant was removed and transferred to spots on silica gel plates and used for TLC The lipid extracts were applied as repeated spots allowing for drying in between The diameter of the spots did not exceed 6 mm Paired unidimensional runs were made at least three times for each preparation using a 0 25 mm layer of silica gel H (Merck Darmstad) In all experiments a mixture of *n* butanol glacial acectic acid water (125 45 37) was used as solvent After 2–3 hours the plates were oven-dried at 75 °C for 30 min allowed to cool and then sprayed with a mixture of 2 vol of 60% H_2SO_4 (v/v) and 1 vol of freshly made 0 1% orcinol in water

Serotyping

Twenty two »Kalmar« isolates obtained from 19 of the 29 pigs were examined serologically a m *Schaefer* (15) in order to determine whether *M avium* serotypes 1–3 could be recovered The test sera were kindly supplied by Dr *J Kenneth McClatchy* (National Jewish Hospital and Research Center Denver Colorado USA) and represent serotypes 1 and 3 (titres 1 160–320) and 2 (1 80–160)

RESULTS

Isolates of »Avium-Like« Mycobacteria from Animals in Sweden

Table 2 shows the results of the three year (1974–76) investigation of »avium like« mycobacteria in domestic and wild animals ALM were isolated from 385 out of 549 pigs and from 5 out of 33 cattle The bacteria were also found in one out of nine horses two out of six sheep and five out of 21

TABLE 2 *Occurrence of »Avium Like« Mycobacteria in Animals in Sweden*

Species	No of cases examined	No of cases positive
Cattle	33	5
Pigs	549	385
Horses	9	1
Sheep	6	2
Mink	21	5
Fowls	27	20
Pheasant	4	3
Peacocks	3	2
Sparrow hawks	2	2
Owls	4	1
Rough legged buzzards	1	1
Kestrels	1	1
Common cranes	2	1
Grey lag geese	1	1
Mute swans	1	1

* Obtained from the Central Veterinary Laboratory Weybridge England

TABLE 1 *Origin of Isolates from Pigs (»Kalmar« Isolates)*

Isolate no	Isolated from	Country	Obtained from
1	submandibular lymph node	Sweden	fresh culture
2[a] ┐	submandibular lymph node	»	»
3 ┘	kidney	»	»
4	submandibular lymph node	»	»
5	submandibular lymph node	»	»
6 ┐	submandibular lymph node	»	»
7 ┘	bronchial lymph node	»	»
8	submandibular lymph node	»	»
9	submandibular lymph node	»	»
10	lung lymph node	»	»
11 ┐	submandibular lymph node	»	»
12 │	liver	»	»
13 │	lung	»	»
14 ┘	lung lymph node		
15 ┐	liver	»	»
16 ┘	lung	»	»
17 ┐	lung lymph node	»	»
18 ┘	submandibular lymph node	»	»
19 ┐	lung lymph node	»	»
20 ┘	submandibular lymph node	»	»
21	submandibular lymph node	»	»
22 ┐	lung	»	»
23 ┘	submandibular lymph node	»	»
24 ┐	lung	»	»
25 │	kidney	»	»
26 ┘	submandibular lymph node	»	»
27	submandibular lymph node	»	»
28 ┐	submandibular lymph node	»	»
29 │	lung	»	»
30 │	liver	»	»
31 ┘	kidney	»	»
32	submandibular lymph node	»	»
33	submandibular lymph node	»	»
34	submandibular lymph node	»	»
35	submandibular lymph node	»	»
36 ┐	lung	»	»
37 │	lung lymph node	»	»
38 │	liver	»	»
39 ┘	kidney	»	»
40 ┐	lung	»	»
41 │	liver	»	»
42 │	lung lymph node	»	»
43 ┘	kidney	»	»
44 ┐	submandibular lymph node	»	»
45 ┘	lung lymph node	»	»
46	lung	»	»
47	lung	»	»
48	submandibular lymph node	»	»
49	submandibular lymph node	»	»
50	submandibular lymph node	»	»
8551	– (pig)	England	NCTC
M 80 (A 1)	liver (fowl)	»	Eric Boughton Weybridge
M 5 (A 2)	liver (fowl)	»	»
M 8 (A 3)	liver (fowl)	»	»

[a] Numbers grouped in square brackets represent isolates from the same animal

A B C

Fig 1 Unidimensional chromatograms of *M avium* strains isolated from fowls designated A 1 (A) A 2 (B) and A 3 (C) on the basis of their lipid composition See text for TLC conditions used

Seroagglutination

Of 22 ›Kalmar‹ isolates investigated one belonged to serotype 1 four to serotype 2 and nine to serotype 3 (Table 5) The NCTC strain 8551 belonged to serotype 2 Six strains failed to agglutinate any of the sera tested and one strain showed spontaneous agglutination

TABLE 4 *Lipid Typing of 50 Kalmar Isolates*

Isolate no	Lipidtype
19 20 33 M 80	A 1
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 18 23 24 25 26 27 32 34 35 37 38 39 41 42 46 47 48 49 50 M 5 NCTC 8551	A 2
28 29 30 31 M 8	A 3
17 21 22 36 40 43 44 45	Not typable

TABLE 5 *Serological Typing of 22 ›Kalmar Isolates*

Seroytype	Isolate no
1	46
2	2 6 9 17 NCTC 8551
3	5 10 20 26 30 31 32 48 49
others[a]	11 22 27 33 34 47
A[b]	21

a Not belonging to *M avium* serotypes
b Spontaneous agglutination

DISCUSSION

In recent years »avium like‹ mycobacteria have been isolated from a whole range of animals in Sweden but especially from pigs (Table 2) The occurrence of these infections in both domestic and wild animals emphasizes their possible role as reservoir and vector for spread among and from animal populations The findings in wild birds demonstrate the possibility of uncontrolled spread over wide areas

The more frequent isolation of ALM from the fowls investigated than from pigs and cattle reflects differences in the macroscopical picture i e in the fowl a typical disseminated tuberculous infection occurs whereas in the others the lesions may be confused with other subclinical diseases

The difficulties of classifying the species within the group of ALM have been described in other studies (2 3 4 5 6 8 12 15 18 19 20 21 22) In a co-operative study on slowly growing non pigmented mycobacteria by the International Working Group on Mycobacterial Taxonomy (11) some of the participants suggested that *M intracellulare* should be reduced to a synonym of *M avium* while others considered that the name *M intracellulare* should be retained It should be mentioned however that it is a long-standing proposal that differentiation between these two taxa should be obtained by the agglutination method devised by *Schaefer* (15) In 1973 *Wolinsky & Schaefer* (23) suggested a new scheme for numbering mycobacterial serotypes where strains of serotypes 1 2 and 3 are classified as *M avium* and serotypes from 4 and onwards are classified as *M intracellulare*

In order to classify the isolates within Runyon s group III by biochemical methods three tests often

TABLE 3 *Biological and Biochemical Properties of 50 »Avium Like« Mycobacteria Isolated from Pigs (»Kalmar« Isolates)*

Isolate no	Runyon group	Growth at 45 °C	Virulence for chicken	Arylsulphatase activity		Tellurite reduction within 3 days	Tween 80 hydrolysis	
				day 3	day 14		day 12	day 20
Subgroup A 3 4 7 8 9 10 11 12 13 14 15 16 17 18 19 22 23 24 25 26 27 28 29 30 31 33 34 35 36 37 38 39 40 41 42 43 44 45 47 50 NCTC 8551 M8 M5 M80	III	+	+	–	–	+	–	–
Subgroup B 5 20 21 32 48	III	+	+	+ a)	+	+	–	–
Subgroup C 1 2 6	III	+	+	–	–	–	–	–
Subgroup D 46 49	III	–	+	–	–	+	–	–

a) One isolate No 21 was arylsulphatase positive after 3 days

mink ALM were also isolated from wild and domestic birds

Biological and Biochemical Examination

According to their biological and biochemical properties »Kalmar isolates nos 1–50 were divided into subgroups A–D (Table 3) All isolates could be assigned to Runyon s group III (non photochromogenic) They were *pathogenic* for chicken i e the animals became moribund or died within 2–8 weeks and on autopsy specific lesions [illegible] spleen l ver lungs [illegible] as able to [illegible] out of 50 strains grew at 45 °C and reduced tellurite but showed no arylsulphatase activity The five strains *in subgroup B differed from* those in subgroup A in that one culture was positive in the arylsulphatase test after three days and a further four strains after *14 days* Those isolates which were *negative in* tellurite reduction (three isolates) were assigned to [illegible] pigs the isolates obtained from each [illegible] the same subgroup but in three they were assigned to different groups

TLC Lipid Patterns

The TLC lipid patterns of the three fowl strains designated A 1 A 2 and A 3 are illustrated in Fig 1 The bacterial lipid fractions were observed as distinct brownish spots on the plates The results of the 50 isolates studied by thin layer chromatography are shown in Table 4 Three of the 50 porcine isolates *yielded chromatograms which appeared to* be identical with that of A 1 35 strains elicited patterns similar to that of A 2 four of the remaining 13 *strains showed similarity to* A 3 Eight of the strains displayed various patterns which could not be typed Five of these were difficult to interpret because of the indistinct spots obtained even when large numbers of bacteria were *extracted and used in the test In eight out of twelve* pigs the isolates recovered from each pig belonged to the same lipid type whereas in four they *displayed various patterns some of which could not* be typed

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applied in the characterization of mycobacteria, viz Tween 80 hydrolyses, tellurite reduction, and arylsulphatase activity, were used in addition to growth at 45 °C and chicken virulence tests The Tween 80 hydrolysis test (20) helps in differentiation of clinically significant and saphrophytic isolates within Runyon groups II and III *Kilburn et al* (7) observed that *M avium-intracellulare* strains showed distinct tellurite reduction within a period of three days, whereas most other slowly-growing, non-pigmented mycobacteria were not positive until six to ten days It has been proposed that the arylsulphatase test is of use in distinguishing between *M avium* and *M intracellulare*, though conflicting results have been reported (14)

As is obvious from Table 3, where the isolates have been divided into four subgroups, 40 out of 50 ALM isolates (subgroup A) showed a virtually identical pattern in the tests, which tallied completely with the reference strains (M5, M8, M80, and NCTC 8551) The discrepancies found with the remaining 10 strains occurred in the arylsulphatase and tellurite reduction tests and growth at 45 °C (subgroups B, C, D) The arylsulphatase activity of the five strains in group B differed in that one isolate showed a strongly positive reaction after three days, whereas the others were positive only after 14 days Further studies are needed to evaluate this phenomenon It has been claimed (14) that the *M intracellulare* strains are positive in the arylsulphatase test more often than *M avium* However, in this study all isolates in subgroup B belonged to *M avium* serotype 3, except for one which showed spontaneous agglutination Three isolates (subgroup C) which were negative for tellurite reduction up to three days are difficult to interpret, though their marked virulence for chicken and their ability to grow at 45 °C makes it possible to exclude most other non photochromogenic mycobacteria from *M avium intracellulare* The two isolates in subgroup D differed from those in group A in their inability to grow at 45 °C Similar results were found by *Juhlin* (6) with certain isolates classified as *M avium*

Accordingly, the biological and biochemical tests used in the present study not only classify the 50 examined ALM isolates in biotypes (A–D) but might also permit them to be assigned to the *M avium-intracellulare* complex

The differences revealed by the thin-layer chromatograms did not correlate with the biological and biochemical properties set out in Table 3, but seemed to show a more complex pattern *Marks & Jenkins* (10) and recently *Sehrt et al* (16) have pointed out the possibility of differentiating between *M avium* serotypes and certain serotypes of *M intracellulare* by TLC However, in the present work, it would appear that among the isolates belonging to lipid type A2, there are some which do not belong to *M avium* serotypes

It appears from the subgrouping used in this study that certain isolates obtained from one and the same animal were assigned to different subgroups, not correlated to the site of sampling This was also the case with some isolates in TLC, which revealed differing lipid patterns within the same animals Further studies are needed to evaluate this phenomenon

In the present study, certain »Kalmar« isolates (14 out of 22 examined) (Table 5) belonged to true *M avium* serotypes (15), whereas the remaining eight isolates might be assigned to *M intracellulare* *M avium* serotype 3 was the most common which is in contrast to results obtained in Denmark (2, 3) and West Germany (12) However, in the USA the serotypes of *M avium* which cause tuberculosis in swine vary from state to state (17), and thus it cannot be excluded that such variations might also exist within Sweden, and between Sweden and other countries

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O-ANTIGENIC CROSS-REACTIVITY IN *FUSOBACTERIUM NUCLEATUM*

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Hofstad T Skaug N & Bjørnland T O antigenic cross reactivity in *Fusobacterium nucleatum* Acta path microbiol scand Sect B 87 371–374 1979

Lipopolysaccharides (LPS) of the same chemotype isolated from seven strains of *Fusobacterium nucleatum* were examined by indirect haemagglutination and inhibition of haemagglutination in unabsorbed and absorbed antisera. Four common major antigenic specificities were detected and two of the LPS examined contained antigenic specificities not shared with any of the other LPS In addition a few weak cross reactions were observed also with some LPS of other chemotypes

Key words *F nucleatum* lipopolysaccharides O antigenic specificity O-antigenic cross reactivity

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Lipopolysaccharides (LPS) of *Fusobacterium nucleatum* are endotoxins (10 11) In addition to a typical lipid A (4) they have in common glucosamine glucose L *glycero* D *manno* heptose and 3 deoxy 2 keto-octulosonic acid (KDO) but differ with respect to other sugars (3) Based on the presence or absence of galactose rhamnose or D *glycero* D *manno* heptose LPS isolated form 20 strains of *F nucleatum* could be classified into six chemotypes (I–VI) (3)

The LPS of *F nucleatum* show a high degree of serological specificity (2 9) *Kristoffersen et al* (9) employing inhibition of indirect haemagglutination found four antigenic specificities in LPS from three strains of *F nucleatum* (strains F1 Fev 1 and ATCC 10953) Each LPS had its own specificity and one specificity was shared by the LPS of strains F1 and Fev 1 The present study concerns the O antigenic cross reactivity of chemotype II LPS and the elaboration of a preliminary typing system for *F nucleatum* based on inhibition of indirect haemagglutination

MATERIALS AND METHODS

Organisms

The origins of the *F nucleatum* strains Fev 1 F5 F7 F8 F13 F14 F15 and of other *F nucleatum* strains included in the study have been reported previously (3) Cultivation was performed in 500 ml screw cap bottles filled to the top with an enriched fluid medium based on tryptone (3)

Lipopolysaccharide

Washed organisms were extracted with phenol water (12) at 20 °C and the LPS were purified from the water phases by ultracentrifugation (100 000 × *g* 90 min) and treatment with DNase and RNase (8)

Serological Methods

Antisera were raised in rabbits by immunization with whole cells (5) Sensitization of sheep erythrocytes with NaOH treated LPS indirect haemagglutination (IHA) and inhibition of IHA were carried out as described previously (7) IHA inhibition was performed with unabsorbed antiserum or with antiserum absorbed with whole microbial cells using nine volumes of cell suspension (OD 2 0 at 600 nm) for absorption of one

RESULTS

'ntisera were produced against the *F nucleatum* rains Fev 1 F5 F7 F8 F13 F14 and F15 With e exception of antiserum to strain F5 all of them ontained antibodies reacting with two or three of e heterologous LPS (Table 1) The results of the HA tests indicated a similarity in O antigenic pecificity of LPS prepared from strains Fev 1 F8 nd F15 and of the LPS of strains F7 F13 and 14 This was confirmed by inhibition of IHA esults not given) and by absorption experiments Table 2) The results given in Table 1 and Table 2 ere obtained with the same (unabsorbed or bsorbed) antisera The titres listed in Table 2 were e same after reabsorption of the antisera

According to the results of the absorption xperiments at least five different antigenic specifi ities were present in the LPS preparations from trains Fev 1 F7 F8 F13 F14 and F15 These pecificities are hereinafter termed O antigens 1–5

O-antigen 1 Absorption of antiserum Fev 1 with train F8 removed the haemagglutinating antibodies n this antiserum to LPS of strains Fev 1 F8 and F15 (Table 2 row (3)) Absorption of the same ntiserum with strain F15 caused a four to eight fold reduction in the antibody titre to sheep erythrocytes sensitized with LPS of strain Fev 1 or F8 (Table 2 row (4)) Absorption of antiserum F8 with strains Fev 1 or F8 removed all haemaggluti nating antibodies except those reacting with sheep erythrocytes sensitized with LPS of strain F5 (Table 2 row (1...

... isolated from the strains Fev1 F8 and F15 have one antigenic specifity (or more) in common

O antigen 2 Following absorption of the antisera to strains Fev 1 and F8 with strain F15 antibodies reacting with sheep erythrocytes sensitized with LPS of strain Fev 1 or F8 were still present in the antisera (Table 2 rows (4) and (15)) This shows that LPS isolated from strains Fev 1 and F8 share at least one antigenic specificity not present in LPS of strain F15

O-antigen 3 Absorption of antiserum to strain F7 with strain F13 and to antiserum to strain F13 with strains F7 ...

ting s ...

of the ...

2 rc ... Consequently the LPS isolated from strains F7 F13 and F14 have one antigenic specificity (or more) in common

O-antigen 4 After absorption of the antisera to strains F7 or F13 ... from strain

TABLE 3 *Test Systems (IHA) for Demonstration of the O Antigens 1–5 in Strains of* F nucleatum *Unknown LPS are to be Used as Inhibitors*

O antigen	Antiserum (4 agglutinating units)	Sensitizing antigen (25 µl/ml)
1	Anti Fev 1	LPS F15
2	Anti Fev 1 abs F15	LPS F8
3	Anti F13	LPS F14
4	Anti F13 abs F14	LPS F7
5	Anti F14 abs F13	LPS F14

F14 antibodies that reacted with sheep cells sensitized with LPS of strain F7 or of strain F13 were still present in the antisera (Table 2 rows (10) and (19)) This shows that LPS of strains F7 and F13 share one or more antigenic specificities not present in LPS of strain F14

O antigen 5 After absorption of antiserum to strain F14 with LPS of strains F7 or F13 antibodies that reacted with sheep cells sensitized with the homologous LPS were still left in the serum (Table 2 rows (22) and (23)) Consequently LPS F14 has one specificity in addition to the specificity shared with LPS of strains F7 and F13

Based on interpretation of the results of the absorption experiments test systems for demonstra tion of the antigens 1–5 were constructed The components of each system are shown in Table 3

The presence in antiserum to strain F5 of antibodies that did not react ...

absorbed antiserum to strain F8 to LPS of strain F5 (Table 2 rows (12) (13) and (15)) is difficult to explain They were not present in pre immune serum LPS of strain F5 may share an antigenic specificity with LPS ...

... examined for antibodies reacting with sheep erythrocytes sensitized by LPS isolated from strains F1 and ATCC 10953 The sheep cells sensitized with LPS of strain F1 agglutinated weakly in the antisera to strains Fev 1 F7 F13 and F15 None of the antisera contained antibodies reacting with sheep cells sensitized with LPS of

TABLE 1 *Titres in IHA Tests of Antibody to LPS in Antisera to* F nucleatum *Strains Fev 1 F5 F7 F8 F13 Fl*
F15

Sensitizing antigen (25 µg/ml)	Anti Fev 1	Anti F5	Anti F7	Anti F8	Anti F13	Anti F14	Ant F1
LPS Fev 1	2560	–	–	640	–	–	128
LPS F5	–	640	–	40	–	–	–
LPS F7	–	–	2560	–	10240	160	–
LPS F8	2560	–	–	2560	–	–	64
LPS F13	–	–	1280	–	640	40	–
LPS F14	–	–	1280	–	2560	1280	–
LPS F15	1280	–	–	640	–	–	64

– titre less than 20

TABLE 2 *Absorption of antisera to* F nucleatum *strains Fev 1 F5 F7 F8 F13 F14 and F15 with various*
Effect on titres in IHA

Antiserum	Strain used for absorption	Source of LPS used for sensitization Fev 1	F5	F7	F8	F13	F14	F1
(1) Anti Fev 1	PBS control	2560			2560			256
(2)	Fev 1	–			–			–
(3)	F8	–			–			–
(4)	F15	640			320			–
(5) Anti F5	PBS control		1280					
(6)	F5		–					
(7) Anti F7	PBS control			2560		2560	2560	
(8)	F7			–		–	–	
(9)	F13			–		–	–	
(10)	F14			320		160	–	
(11) Anti F8	PBS control	640	40		1280			64
(12)	F8	–	40		–			–
(13)	Fev 1	–	40		–			–
(14)	F5	160	–		1280			16
(15)	F15	160	40		80			–
(16) Anti F13	PBS control			10240		320	5120	
(17)	F13			–		–	–	
(18)	F7			–		–	–	
(19)	F14			320		320	–	
(20) Anti F14	PBS control			80		80	640	
(21)	F14			–		–	–	
(22)	F7			–		–	40	
(23)	F13			–		–	640	
(24) Anti F15	PBS control	640			640			640
(25)	F15	–			–			–
(26)	Fev 1	–			–			–
(27)	F8	–			–			–

– titre less than 20

PHAGOCYTOSIS OF ^{32}P-LABELLED *ESCHERICHIA COLI* BY HUMAN POLYMORPHONUCLEAR CELLS (PMN)

Effect of Different Sera and PMN with Reference to the ABO Blood Group System

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Melby K Phagocytosis of ^{32}P labelled *Escherichia coli* by human polymorphonuclear cells (PMN) Effect of different sera and PMN with reference to the ABO blood group system Acta path microbiol scand Sect B 87 375–377 1979

A study on the interaction of different sera and polymorphonuclear cells (PMN) with reference to the ABO blood group system on the phagocytosis of a radiolabelled strain of *E coli* is reported Using untreated sera O cells were found to be the least sensitive and AB cells the most sensitive to reduction in phagocytic activity No reduced phagocytic capability relative to the different sera used was observed when heat inactivated sera were applied Aspects of these results are discussed

Key words Polymorphonuclear cells phagocytosis ABO blood groups *E coli*

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It has previously been demonstrated that a method for [illegible]

[illegible]

on the elimination phase of the process of phagocytosis imply a much longer period of interaction between PMN the serum factors involved and the bacteria (8)

Transplantation of an organ requires compatibility within the ABO blood group system It is likely therefore that also ABO incompatibility will interfere with the phagocytic process and this possibility has been studied here

MATERIALS AND METHODS

Pol[illegible]

fr[illegible]

(9) [illegible] were used

Serum

All sera were obtained commercially and typed at the Red Cross Blood Center Oslo The ABO types used were A_1 B A_1B and O The sera were stored in small aliquots at −80 °C until use

Bacterium

The test bacterium previously identified as *E coli* according to Bergey s Manual(1) has been used in other studies on phagocytosis (8 9 12) The strain was stored in lyophilized state at 5 °C and was also kept in subculture on lactose bromthymol blue agar plates Serological studies of the bacterium were performed with antisera obtained from Behringwerke AG Marburg Lahn W Germany and from Wellcome Reagents Ltd Beckenham England

Phagocytosis Experiments

Monolayers of glass adherent human PMN obtained according to the procedure of *Midtvedt & Melby* (9) were incubated in tissue culture tubes for one h with Krebs R[illegible]

strain ATCC 10953 Some of the antisera contained antibodies reacting with sheep erythrocytes sensitized with LPS of chemotypes III, IV or V, isolated from eight other oral strains of *F nucleatum* When examined for inhibition of IHA none of these gave positive reactions in the test systems for O antigens 1 to 5

DISCUSSION

The results of the present study confirm the presence in *F nucleatum* of O antigenic specificity, as suggested earlier by *de Araujo et al* (2) and *Kristoffersen et al* (9) The seven chemotype II LPS contained both shared and strain specific antigens This serological diversity indicates variation in the structural arrangement of the sugars present in the LPS A few cross reactions between these LPS and LPS of other chemotypes were also observed The cross reactivity of LPS prepared from strains Fev 1 and F1 described previously (9) was thus confirmed

The study demonstrates that test systems based on inhibition of IHA may be used for identification of *F nucleatum* O antigens Such identification may prove valuable in immunochemical studies of LPS of selected strains of *F nucleatum*

Serotyping of a microorganism based on its O antigens is of value in ecological and epidemiological studies but also for identification of certain clinical isolates The invariable presence of *F nucleatum* in large numbers in suppurative or necrotic oral lesions in man such as Plaut Vincents angina and acute necrotizing ulcerative gingivitis and the occasional isolation of *F nucleatum* in pure culture from other suppurative lesions and from blood show that the organism is able to invade human tissue This is supported by the presence in normal human serum of antibodies agglutinating sheep erythrocytes sensitized with LPS of *F nucleatum* (6) and the presence in human gingival tissues of antibodies reacting with extracts of oral fusobacteria (1) It would be of interest to study whether the invasive property is restricted to strains of certain serotypes However such an investigation would necessitate a larger number of defined O antigens and additional test systems

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untreated serum a major reduction in phagocytic capacity was observed with O serum Attachment of antibodies to the PMN surface may block the function of the phagocytic cell either by covering the surface of the cells or by stopping the ingestion because of consumption of complement The method used in this study is dependent on complement for maximal operation (9) This is in accordance with the findings of *Menzel et al* (7) using human PMN and *E coli* By interaction between bacteria and serum or cells and serum in our system the concentration of complement may be reduced If so reduced ingestion of the radiolabelled bacterium may result

When heat inactivated serum was tested a ten fold reduction in the uptake of radiolabelled *E coli* was observed This is in accordance with the findings of *Men el et al* (7) In our study the reduction of phagocytic capacity was not statistically significant However using B serum a stimulation of the phagocytic capacity by AB cells and O cells was observed Similar results were obtained when A serum was used This effect might be due to the presence of anti B antibodies combining with the O 86 antigen of the bacterium thus making the organism more susceptible to phagocytosis The effect of immunoglobulins on the phagocytosis has been studied by *Menzel et al* in a comparable system (7) They report that purified specific IgM has no increasing effect on the phagocytosis In the presence of complement however IgM was very effective On the other hand IgG increased the ingestive capability without the presence of complement *Cunningham et al* (2) have demonstrated that the addition of specific antiserum to a rough strain of *Salmonella typhimurium* results in increased phagocytosis of the bacterium They correlated their findings with the observation of different contact angles between the bacterium and monolayer of PMN when examined with and without serum added to the fluid phase

With B and O serum the PMN from donors of A and AB blood groups might be coated with anti A PMN have Fc receptor (7 11) and the use of serum may interfere with the process of phagocytosis without the presence of complement

Full compatibility with regard to the ABO blood group system seems to be essential when long term effects during the process of phagocytosis are to be studied The use of incompatible sera would generally lead to a reduced uptake of radiolabelled *E coli* This difficulty may be overcome in part by the use of O PMN and AB serum However the possibility also exists that other blood group incompatibilities may interfere Therefore studies on the elimination phase should preferably be performed on PMN and serum from the same person To avoid interaction between natural antibodies and the test strain used in this investigation the donor should have blood group AB or B

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The standard period of incubation of PMN test bacterium and serum was 15 min The monolayer was then washed four times with chilled KRG to remove non ingested and non adherent bacteria All experiments were performed twice with three parallels and serum free controls were always included Phagocytic activity was estimated as described in detail previously (9)

Two different studies were performed one with untreated and the other with heat inactivated sera (30 min at 56 °C)

Statistical Analysis

The Wilcoxon White two sample rank test (6) was used to evaluate the difference in the phagocytic capacity of PMN from untreated or heat inactivated sera

RESULTS

The test strain was shown by slide agglutination to react with antiserum from either of the suppliers directed against 0 86 B 7

The results of the phagocytosis studies are expressed as percentage uptake of radiolabelled *E Coli* when full compatibility between cells and sera within the ABO system was present The results are given in Table 1 & 2 A percentage lower than 100 indicates a reduced and a percentage over 100 an increased phagocytic capacity

Using PMN from a person with blood group O a major reduction was seen in the presence of B serum (anti A present) Using cells from an AB donor a reduction to approximately 50% was recorded when the serum used was incompatible (A B and O) This difference was statistically significant (p < 0 01) Using cells of blood group A or B some *reduction was recorded mostly by the use of O* serum (anti A and anti B present) against B cells (p < 0 01) The use of A cells and B serum also *revealed a reduced ingestive capability* (p < 0 01) (Table 1)

The other part of the study was performed in a similar way but using heat inactivated sera No statistically significant reduction in phagocytic capacity was observed However using either AB *cells or O cells there was an increased uptake of the* bacterium *This difference was statistically signifi* cant (p < 0 01) when testing O cells and B serum but questionable when B serum and AB cells were tested (0 05 < p < 0 1) (Table 2)

TABLE 1 *Effect of Different Untreated Sera on the Phagocytosis of Radiolabelled* E coli *by Human Polymorphonuclear Cells*

Blood group of serum donor	Blood group of cell donor Percentage phagocytic activity[a]			
	A	B	AB	O
A	100	84	54_b	91
B	80_b	100	61_b	80_b
AB	96	105	100	91
O	75_c	55_b	42_b	100

[a] The values obtained when full compatibility was present were set as 100%. *The other values are the* arithmetic means from six observations

b − p < 0 01

c − p < 0 05

TABLE 2 *Effect of Different Heat Inactivated Sera on the Phagocytosis of Radiolabelled* E coli *by Human Polymorphonuclear Cells*

Blood group of serum donor	Blood group of cell donor Percentage phagocytic activity[a]			
	A	B	AB	O
A	100	118	118	119
B	107	100	128_d	138_b
AB	82	98	100	96
O	90	117	93	100

[a] See legend to Table 1

b = p < 0 01

d = 0 1 > p > 0 05

DISCUSSION

The results show the importance of compatibility within the ABO system when human cells and sera are examined for phagocytic capacity The method has been established by the use of standard pooled human serum with reference to the ABO blood *group system* However the problem of incompatible antibodies might be present though at a generally standardized level when the same serum is used within a group of experiments *Knowing* that the PMN *possess the same antigens of the* ABO types as the red cells (3 5) this problem could be minimized by the use of O cells

Another problems is the nature of the bacterium *The bacterium used reacts with antisera directed* against *E coli* 0 86 B 7 The 0 86 antigen has been shown to cross react with antibodies to the *blood* group B substance (4 10) Thus natural antibodies which are *primarily of the IgM class occur in* human A and O sera The antibodies may combine with the bacterium and/or the PMN Using

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MULTIPLE SCLEROSIS AND COMMON VIRAL INFECTIONS IN ICELAND

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Gudmundsdottir S Multiple sclerosis and common viral infections in Iceland Acta path microbiol scand. Sect. B 87 379–384 1979

Sero-epidemiological studies on a few common virus infections including measles rubella mumps and varicella zoster were carried out on patients with multiple sclerosis (MS) and controls matched for age sex and residences since birth The frequency of antibodies against measles was signigicantly higher in the MS patients Where measles preceded the onset of MS the time interval varied from 3 to 32 years In two cases known MS patients contracted measles 9 and 3 years after their onset of MS Furthermore three MS patients were vaccinated against measles as adults Two of these took part in a WHO measles vaccine trial in 1962 18 and 6 years after the onset of MS Both of them were seronegative prior to vaccination

Key words Multiple sclerosis common viral infections Iceland

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Much work has been carried out in recent years to ascertain whether multiple sclerosis (MS) is a viral infection (5 17) Many workers have found elevated measles antibody values in MS patients as compared to matched control groups (1 4 15 20 22)

Iceland is a country which is a high risk zone for MS (7 8 9 10 16) The population numbers approximately 200 000 Many common viral infections cause clear and well-defined epidemics Between epidemics the causal agents disappear from this population which is not big enough to keep them active at all times Exceptions from this rule are viruses belonging to the herpes group and perhaps mumps (21)

The work reported here was carried out to see whether in this rather isolated population matched sero-epidemiological studies on a few common virus infections including measles rubella, mumps and varicella zoster would throw any further light on the possible relationship between these infections and MS

Clinical and Epidemiological Studies on MS in Iceland

Studies on the clinical aspects and epidemiology of MS in Iceland over a period of 35 years were carried out by *Gudmundsson* (7 8 9 10) His main results covering two adjacent 10 year periods are summarized in Table 1 These show that the age at onset was significantly higher during the period 1956–1965 On the other hand the lower figures for incidence and risk during this period are possibly biased by higher age at onset and a shorter period of observation

The ratio male/female of definite and probable MS cases encountered during the period 1958–1972 was 50 79 (0 62)

There seems to be an accumulation of cases in Eastern Iceland In 9 families out of 129 more than one patient was found Thus familial incidence was 7% when calculated by number of families The average annual death rate from 1946–1969 was 0 9%

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MULTIPLE SCLEROSIS AND COMMON VIRAL INFECTIONS IN ICELAND

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Sero-epidemiological studies on a few common virus infections including measles rubella mumps and varicella zoster were carried out on patients with multiple sclerosis (MS) and controls matched for age sex and residences since birth The frequency of antibodies against measles was signigicantly higher in the MS patients Where measles preceded the onset of MS the time interval varied from 3 to 32 years In two cases known MS patients contracted measles 9 and 3 years after their onset of MS Furthermore three MS patients were vaccinated against measles as adults Two of these took part in a WHO measles vaccine trial in 1962 18 and 6 years after the onset of MS Both of them were seronegative prior to vaccination

Key words Multiple sclerosis common viral infections Iceland

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TABLE 1 *Results of Clinical and Epidemiological Observations on MS in Iceland During Two 10-Year Periods (10)*

		1946–1955	1956–1965
Years between onset and diagnosis of MS		12	5 6
Prevalence at the end of period per 100 000 inhabitants	Both sexes	52 7	52 3
	Males	39 8	44 0
	Females	65 7	60 7
Age at onset of MS		26 5	31 6
Incidence per 100 000 inhabitants		3 1	1 9
Risk at age 10–50 years		5 3	3 4

MATERIALS AND METHODS

MS Patients

Professor *A R Gudmundsson** kindly provided the writer with a list of all known patients with MS alive in Iceland at the time of this study (1974–1976) and gave the author access to all his journals and records He and the writer collected blood samples from most of his MS patients and from MS patients in institutions for the disabled in Reykjavik and vicinity District physicians in various rural areas collected specimens from their MS patients Thus it was possible to obtain samples from 70 patients 31 males and 39 females which was approximately 70 % of definite and probable MS patients alive at that time Each patient was questioned about his places of residence since birth and asked to give a detailed history (including time and place) of the following viral diseases measles mumps rubella varicella herpes zoster herpes simplex lesions poliomyelitis Bornholm s disease meningitis and encephalitis

Most of the patients were able to indicate whether they had had the first five diseases but had difficulty in remembering when they contracted the illness Measles was by far the best remembered since five out of every six patients gave definite answers but only one out of two patients could give definite answers regarding the other four diseases

Controls

The writer felt that for comparison of serological data from MS patients a carefully matched control group was needed It was decided therefore to find a healthy individual to match each patient This control person had to be of the same sex and the same age (± 3 years) as the MS patient Furthermore it was considered very important that such person had the same birthplace and had lived for an equally long time in the place or places where the MS patient had lived prior to the onset of MS

In Iceland the importance of matching for birthplace and place(s) of residence is obvious Epidemics of common infections usually affect the densely populated part of Iceland the capital Reykjavik and vicinity They do not necessarily spread much further and some areas always escape Controls selected as described above were thought to rule out the possibility that serological differences between the MS patients and the control group could be accounted for by differences in age sex and/or places of birth and residences

In order to find suitable controls the writer questioned blood donors other available healthy individuals and people hospitalized for minor operations Those who matched a patient were accepted as controls District physicians who provided sera from MS patients were usually able to find controls for their patients By these means it was possible to match 42 patients 19 males and 23 females

Collection and Storage of Specimens

Blood specimens were collected into sterile vacuum containers Sera were separated by centrifugation at 3000 rev/min for 10 minutes from Reykjavik and vicinity a few hours after collection Specimens sent from rural areas were never more than two days old when separated Sera were kept frozen at − 25 °C in sterile vials

Test Methods

Complement fixation (CF) tests were carried out against measles mumps and varicella zoster (V Z) viruses cytomegalovirus (CMV) respiratory syncytial virus (RSV) and parainfluenza viruses I II and III The long fixation method of *Grist et al* was used (6) Sera were diluted in twofold steps from 1 4 to 1 256 Complement haemolysin CF antigens and CF control antigens were obtained from the Flow Laboratories in Scotland Sheep blood was purchased from the Institute for Experimental Pathology at Keldur Reykjavik and kept in Alsever s solution for a period of no longer than 10 days for each batch A 4 + reaction (no lysis of sheep RBC) in the 1 4 serum dilution was the lowest reaction considered positive if there was complete lysis of RBC in the well containing the same serum dilution and control antigen

Haemagglutination inhibition (HI) tests were carried out to detect rubella and measles antibodies All sera tested by the HI methods were pretreated by kaolin (23) and then 50 % solution of the red blood cells was added for absorption of non specific inhibitors The sera were not tested for rheumatoid factor

The rubella haemagglutination (HA) antigen was purchased from the Flow Laboratories in Scotland Pigeon red blood cells from a local colony in this laboratory were used in the test The method was a microtitration carried out as described by the Flow Laboraries except that the antigen antibody binding time was extended from 1 to 1 ' hours (3 24) Titre 1 20 was considered the lowest positive value

* Deceased 1977

The measles HA antigen and monkey (*Cercopithecus aethiops*) red blood cells in 5% solution were purchased from Behringwerke AG in West Germany. Dilutions were made in phosphate buffered saline (PBS) (6) at pH 7.2 and the microtitration method was used. The test was performed by binding together antigen and antibody at 37 °C for 1½ hours plus 1 hour at room temperature (20 °C). After adding 0.5% suspension of monkey red blood cells the plates were incubated at 37 °C for 1½ hours before the test was read. Titre 1:10 was considered the lowest positive value.

RESULTS

... Neither did comparison of each patient with his matched control reveal any significant differences.

Two-thirds of the patients and their controls were tested for CF antibodies against the parainfluenza viruses. The results are shown in Table 3. Antibodies against these viruses were found more often in sera from the patients but the positive titre values were very similar in both groups. The proportion of people lacking antibodies against parainfluenza virus I was very high.

Rubella HI tests did not show significant differences between patients and controls.

Comparisons of MS patients and controls as regards CF and HI antibodies against measles are shown in Table 4.

In the measles CF test the log geometric mean titre (GMT) values were very similar in both groups. On the other hand there was a significant difference between MS patients and controls when distribution of positives and negatives in the measles

TABLE 2 *CF Antibodies against Mumps, RSV, CMV and V Zoster in MS Patients and their Matched Controls (C)*

	Mumps		RSV		CMV		V Z	
	MS	C	MS	C	MS	C	MS	C
Number negative (titre < 1/4)	14	16	4	7	16	11	22	24
Number positive (titre ≥ 1/4)	28	24	37	34	26	31	11	9
	42	42	41	41	42	42	33	33

TABLE 3 *CF Antibodies against Parainfluenza Viruses I, II and III in MS Patients and Matched Controls (C)*

	Para influenza virus I		Para influenza virus II		Para influenza virus III	
	MS	C	MS	C	MS	C
Number negative (titre < 1/4)	18	26	7	12	2	8
Number positive (titre ≥ 1/4)	14	6	22	17	25	19
	32	32	29	29	27	27

TABLE 4 *CF and HI Antibodies against Measles in MS Patients and Matched Controls*

	CF test		HI test	
	MS patients	Controls	MS patients	Controls
GMT (log)	3 8	3 4	5 95	5 05
Number negative[a]	8	18	1	3
Number positive	34	24	41	39
	42	42	42	42

[a] Titre < 1/4 in the CF test
Titre ≤ 1/5 in the HI test

TABLE 5 *Data on Multiple Sclerosis and Measles in 13 MS Patients and Controls*

Pair	Age at onset of MS	Age at time of measles	Years between measles and onset of MS	Titres CF
M–MS	14	23	9 years after onset of MS	1/16
C		Not known		<1/4
F–MS	17	7	10	<1/4
C		1		<1/4
F–MS	18	8	10	1/32
C		11		1/16
M–MS	21	10	11	1/8
C		9		1/16
F–MS	24	Vacc	18 years after onset of MS	1/8
C		Not known		1/4
M–MS	25	17	8	1/16
C		Never had measles		<1/4
M–MS	28	21	7	1/16
C		39		1/4
F–MS	29	26	3	1/8
C		23		1/8
F–MS	31	14	17	1/16
C		13		1/64
M–MS	32	19	13	1/8
C		17		1/16
F–MS	34	21	13	1/8
C		12		1/16
F–MS	36	Vacc	6 years after onset of MS	<1/4
C		Not known		<1/4
F–MS	48	19	29	1/128
C		14		<1/4

M = males F = females C – control Vacc – vaccinated against measles

CF test was compared by the chi square method ($0.01 < p < 0.05$) In the measles HI test the difference between GMT ($\log_2$) of MS patients and their matched controls was almost twofold Some authors consider a twofold difference in GMT significant (22)

When measles antibody titres of individual pairs

pairs did not show such difference the MS patient had a significantly higher titre in 12 pairs and the control in 2 pairs The same applies to 18, 15 and 5 pairs, respectively, when the HI antibody titres were compared Four pairs were excluded three because the patient had been vaccinated against measles and one because the control had never had measles

Table 5 shows examples of individual compari

son of MS patients and their matched co including the age at onset of MS and relation history of measles For those who had m before onset of MS the shortest time between events was 3 years and the longest 32 years

Two patients a father and a daughter both definite diagnosis had natural measles afte onset of MS The father had measles in 1916 a age of 23 9 years after the onset of MS He is seropositive The daughter had measles in 19 years after the onset of MS Blood samples c not be obtained Both father and daughter were and have always lived in Eastern Iceland These patients and a few others not included here similar history of measles infection were kn before this study was carried out (8)

Three patients (females) were vaccinated aga measles after the onset of MS Two of them

TABLE 6 *Measles Epidemics in Iceland 1846–1965*

Years	Cases registered
1846	Not known
1882	Not known
1907–1908	7 397
1916–1917	4 691
1924–1926	6 125
1928 1929	5 319
1935–1937	8 324
1943–1944	7 156
1946 1948	4 796
1951–1952	7 244
1953–1955	8 183
1958–1959	7 102
1962–1964	7 406

part in a WHO measles vaccine trial in 1962 and were tested for measles HI antibodies before administration of the live vaccine (Enders Edmonston B) (11) They were seronegative and reacted to the vaccination with fever rash and seroconversion thus *confirming that they* had never had measles Many seronegative members of their families and neighbours were vaccinated in the same vaccine trial These two women were born in an isolated rural *area in* North East Iceland and have always lived there The older had onset of MS when she was 24 years old which was 18 years before the measles vaccination The younger one had onset of MS when aged 35 which was 6 years prior to the vaccination

The epidemiology of measles in Iceland is well known (21) The first epidemic occurred in 1846 as on the Faroe Islands (19) Dates of the main measles epidemics are shown in Table 6

When patients and controls could give exact information concerning the year they had had measles it could be connected in most cases with known epidemics Most of the earlier epidemics left some rural areas untouched especially the North Eastern and Eastern parts of Iceland where measles vaccination of adults has been carried out extensively since 1962 (11 12) Between epidemics the virus disappears from the Icelandic population Since 1965 measles vaccine has been widely used and the epidemiological pattern is consequently changing

DISCUSSION

The results presented here are in most aspects similar to those of many other authors (1 2 20 22) A difference between MS patients and controls is found as regards measles antibodies The most

measles in this

than controls were positive (titre ≥ 1/4) in the measles CF test but still 19% of the patients were negative The writer is aware of only one paper where the ratio between negatives and positives in the CF tests is mentioned but similar results were obtained in that report (20)

2) If it is assumed that measles inevitably precede the onset of MS it is interesting to note the great variation in time between the two events in these patients (Table 5) the shortest period being 3 years and the longest 32 years It may be worth while to compare this time interval with the fate of a known slow virus in its natural host e g sheep inoculated intracerebrally at the same time with the same amount of visna virus (13 14) In these visna experiments the date of onset of clinical visna differed considerably and varied from 3½ months to 10 years

3) The most interesting point in this work is that some patients did not contract clinical measles before the onset of MS For the two persons who took part in a WHO measles vaccine trial in 1962 after the onset of MS negative results of serological tests prior to the vaccination are available (11)

These serological results seem to be correct since the patients reacted to the measles vaccination with fever and rash during the second week It is generally agreed that sub-clinical measles infections are very rare It was found in the WHO vaccine *trial* in 1962 that the history of those in rural Iceland who denied having had measles was reliable in all cases (11)

The Icelandic experience indicates that MS can occur with high incidence in the virtual *absence of* canine distemper Canine distemper has not been

part of the eastern region where the incidence of MS is highest was *involved* to a small extent in the outbreak of 1941–1942 (18)

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A RUBELLA HAEMAGGLUTINATION INHIBITION TEST NOT REQUIRING REMOVAL OF NON-SPECIFIC INHIBITORS

I Elaboration of the Test

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Haukenes G A rubella haemagglutination inhibition test not requiring removal of non specific inhibitors I Elaboration of the test Acta path microbiol scand Sect B 87 385–389 1979

The rubella haemagglutination inhibition (HI) test can be simplified and the problem of non specific inhibitors circumvented by allowing the haemagglutinin to react with erythrocytes before addition of the serum specimen The elaboration of the test procedure is described and preliminary studies indicate that the new method may prove useful both in the diagnosis of rubella infection and for determination of immunity status

Key words Rubella haemagglutination inhibition test simplification

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The rubella haemagglutination inhibition (HI) test (9) has been used widely for serological diagnosis of rubella infection and assessment of immunity status Several modifications have been proposed to render the test more reliable The main problem is the presence in all sera of non specific inhibitors of haemagglutination (HA) False positive reactions due to incomplete removal of inhibitors have been reported for most methods Moreover false negative reactions due to concomitant removal of antibodies have been claimed to occur when k[illegible] can be modified for the determination of specific IgM One major advantage is that non specific inhibitors do not interfere in these new methods This is quite remarkable since the antigen used is the viral haemagglutinin There is however one main difference in procedure between the HI test and those mentioned above in the latter the viral [illegible] with antibody for the haemagglutinin must be incorrect The only explanation is that the inhibitors act by interfering with the binding of the haemagglutinin to the erythrocytes If therefore the haemagglutinin is allowed to react with the erythrocytes before serum is added non specific inhibitors will not influence the reaction between haemagglutinin and antibody However the risk of spontaneous HA or sensitization of erythrocytes f[illegible]

MATERIALS AND METHODS

Rubella Virus Haemagglutinin

This was obtained from Behringwerke Marburg Lahn West Germany It was a Tween 80 ethylether extract of baby hamster kidney (BHK/21) cells and gave an HA titre of 128 to 256 with the four types of erythrocytes used (see below) Haemagglutinin preparations from other producers (Grand Island Biological Corporation VR 350 DL Wellcome V4 01 Microbiological Associates 30-959I Flow Labs and Orion Diagnostica D 448 and D 868) gave identical results in the HI test

Sera

All sera were specimens sent to the routine laboratory They were examined either in fresh state or after storage at −20 °C and were heat inactivated (56 °C 30 min) before use

Erythrocytes

Sheep erythrocytes were employed for the development of the new HI test They were stored at 4 °C in Alsever s solution for up to one week The working suspension was standardized spectrophotometrically at 542 nm Comparable studies were made using erythrocytes from newly hatched chickens from human cord blood and from human blood group 0 individuals The latter erythrocytes were pretreated with 0 1% trypsin (Crystalline Novo Copenhagen) for one hour at room temperature

Buffers

Kaolin was suspended in borate saline pH 9 0 Otherwise the dilunt was a 25 mM HEPES buffer made isotonic by NaCl and supplemented with 10 mM Ca^{2+} and 4 mM Mg^{2+} In the standard HI rest 0 25% bovine albumin was added to the buffer just before use and the pH was adjusted at room temperature to 6 2 (note that the ΔpK_a/°C of HEPES is −0.014) In the new HI procedure no protein supplement to the buffer was made except for HA titration when 0 0025% gelatine was added

Standard HI Test

Serum diluted 1 10 was absorbed by kaolin (Flow Laboratories Irvine Scotland) at pH 8 5–9 0 (8) and thereafter by erythrocytes according to standard procedures The other reagents were made up in the HEPES buffer described above The final pH in the reaction mixture was 6 5 Four HA units were used Under these conditions a 16 5% immunoglobulin preparation (Kabi Stockholm) gave an HI titre of 640

Floatation Centrifugation

This was performed as described in (4) In brief N... was added to the serum to give a density of 1 30 A N... solution of density 1 25 was placed over the serum After centrifugation at 100 000×*g* for 18 h top ... bottom fractions were examined

EXPERIMENTS AND RESULTS

HI by Antibody Negative Serum

If non specific inhibitors interfere with the binding of haemagglutinin to erythrocytes high HI titres might be expected if an antibody negative serum is added to the erythrocytes prior to the haemagglutinin

Furthermore no HI would be recorded if the haemagglutinin is allowed to react with the erythrocytes beforehand Table 1 shows that this proved to be true

Inhibition of HA of Sensitized Erythrocytes by Antibody Positive Serum

To study the effect of antibody on the agglutination erythrocytes were sensitized with different amounts of haemagglutinin The serum used showed an HI titre of 320 by the standard HI method The checkerboard titration is shown in Table 2 A sub agglutinating dose (0 5 HA unit) does not sensitize the erythrocytes for agglutination by antibody Eight to 16 HA units were required to obtain an indirect HA When 4 and 8 units of haemagglutinin were used antibody inhibited the agglutination as in an ordinary HI rest

Erythrocyte suspensions sensitized by 1 to 32 HA units were examined by microscopy No true agglutination or only an occasional agglutinate of 2 to 4 erythrocytes appeared when doses of up to 8 HA units were used With 16 and 32 HA units

TABLE 1 *Inhibitor Titre of an HI Antibody Negative Serum when the Reactants were Added at Different Times*

Serum + haemagglutinin	30 min	erythrocytes	HI 80
Serum + erythrocytes	30 min	haemagglutinin	HI 320
Haemagglutinin + erythrocytes	30 min	serum	HI <10

TABLE 2 *The Effect of Ant body on Erythrocytes Sensiti ed by D fferent HA Units*

	Serum dilution							
HA units	10	20	40	80	160	320	640	Buffer
0 5	–	–	–	–	–	–	–	–
1	–	–	–	–	–	–	+ +	+ + +
2	–	–	–	–	–	+ +	+ + +	+ + +
4	–	–	–	–	–	+ + +	+ + +	+ + +
8	+ +	–	–	+	+ + +	+ + +	+ + +	+ + +
16	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ +	+ + +
Buffer	–	–	–	–	–	–		–

no agglutination (HI)
\+ to + + + agglutination (HA)

increasing amounts of true agglutinates were found Two mechanisms for the HI are possible antibody may elute the haemagglutinin from the erythrocy tes, or it may cover the haemagglutinin on the erythrocytes To examine this an antiserum against human IgG was added to those reaction mixtures which showed HI with 4 HA units The HI was thereby converted to an indirect HA with visible clumps of erythrocytes thus indicating that IgG was bound to the sensitized erythrocytes

ELABORATION OF THE TEST PROCEDURE

Source of Erythrocytes

Almost identical HA and HI titres as shown in Table 2 were obtained using erythrocytes from sheep newly hatched chicken and human cord blood and with trypsinized human O erythrocytes The test was elaborated with sheep erythrocytes since these were always readily accessible and were less prone to non-specific inhibition with some antibody negative sera A drawback is the need for absorption with the erythrocytes

In contrast to the findings of others sheep erythrocytes could be used without pretreatment be trypsin (6) since this treatment did not increase the sensitivity of the HA test. Moreover although the haemagglutinin was maximally active at pH 6 2 as found with other erythrocytes we did not find it necessary to use HEPES buffer as claimed by others (7)

Sensiti ation of Erythrocytes

The conditions for optimal sensitization were the same as for the HA test *i e* pH 6 2 a temperature of 4 °C and the presence of divalent ions (Mg^{2+} and/or Ca^{2+}) For titration of HA albumin or gelatine had to be added for the sedimentation of the erythocytes Protein was not added when the erythrocytes were sensitized since this increased the tendency to non specific inhibition in the final HI test Erythrocyte concentration giving an absor bance of 0 8 at 542 nm corresponding to about 0 28% proved optimal It is essential that the concentration is measured spectrophotometrically at every set up

The effect of incubation time of erythrocytes and haemagglutinin with regard to cl mp ng of [illegible]

The New HI Procedure

Pilot experiments revealed three procedural con ditions for obtaining optimal results

1 Use of V microtitre plates instead of U plates With the latter an increased tendency to indirect HA at low serum dilution was observed
2 The buffers should not be supplemented with albumin or gelatine as these proteins caused non-specific inhibition with low serum dilu tions Even at high serum dilutions (640) there was enough protein to secure easily read HA patterns
3 Sensitized erythrocytes must be incubated with serum at 37 °C At lower temperatures the antibody titres decreased and the sedimen tation pattern was often difficult to read

Based on the pilot experiments the following procedure is proposed

1 Heat inactivation of serum (56 °C 30 min)

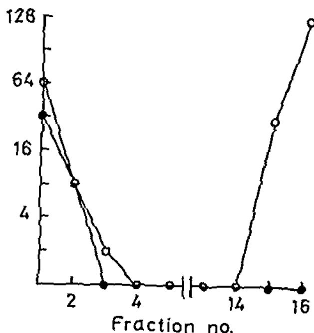


Fig 1 Floatation centrifugation of an HI antibody-positive serum
o——o Standard procedure (except that kaolin treatment is omitted)
•——• New procedure

2 One volume of 4 HA units of haemagglutinin is mixed with two volumes of sheep erythrocytes suspended to give an absorbance of 0 8 The mixture is incubated at 4 °C for about 30 min

3 Serum is diluted 1 10 (25 μl of serum and 225 μl of 10 % sheep erythrocytes) and shaken in an ice-water bath After centrifugation to remove the erythrocytes, serial two fold dilutions of the serum are made in 25 μl volumes

4 Addition of 75 μl of sensitized erythrocytes Two microtitre plates are usually prepared beforehand during incubation for sensitization ready for the addition of sensitized erythrocytes Incubation at 37 °C for 60 min

5 Readings of pattern are made in the usual way, after the 60 min incubation at 37 °C, or after further incubation at 4 °C

Preliminary Results with the New HI Procedure

Floatation centrifugation of an antibody-positi serum showed that the new method measur rubella HI antibody but did not measure no specific inhibitors (Fig 1)

A panel of 561 sera from the routine materi was examined in parallel using the standard and th new HI procedure (Table 3)

There was relatively good correlation between th two methods Sera which differed considerabl were re-examined They were prediluted in tubes and aliquots of each dilution were transferred to th microtitre wells With this procedure, no differen ces of more than a one-titre step were observed Th new method was slightly less sensitive than th standard HI method

Table 3 shows that the titre differences wer greater with high-titred sera The reason for this was that less material was transferred by the microtitre loops when the serum had not been treated with kaolin Using micropipettes instead of loops, the results correlated weel There is no doubt that both methods measured the same antibody

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Studies are in progress comprising a half-year routine material which will be examined by an indirect HA test (»Rubacell«, Abbot Labs) in order to reveal possible false negative and false positive reactions In doubtful cases haemolysis in gel and floatation centrifugation will be performed

TABLE 3 *HI Titre of Serum Specimens by Standard and by New Rubella HI Procedure*

New procedure	Standard procedure <10	10	20	40	80	160	320	640
320	0	0	0	0	1	0	0	0
160	0	0	0	0	0	0	0	0
80	0	0	0	2	15	35	25	6
40	0	2	9	74	88	39	19	5
20	15	12	28	40	15	9	4	0
10	7	2	6	3	0	1	0	0
< 10	86	6	11	0	0	0	0	0

DISCUSSION

The present results show clearly that non specific inhibitors of rubella virus HA do not compete with antibody when the haemagglutinin has reacted with the erythrocyte beforehand

The exact chemical basis for the inhibition is not known. We have shown earlier that all classes of lipoproteins inhibit rubella HA and the inhibitory activity disappears when serum is treated with phospholipase C (2) The inhibitor site of the lipoprotein molecule must therefore reside in the phosphatidyl residue at the C3 position of glycerol i e the hydrophilic part of the phospholipid molecule The haemagglutinin is also a lipoprotein Treatment of the haemagglutinin with phospholipase C has no effect of the HA property or the binding of inhibitors whereas treatment with trypsin destroys the HA property (3) The inhibitors may react with that part of the protein which is responsible for the HA activity or with nearby sites Attempts have been made to block the inhibitory receptors Lecithin blocked the HA activity (2) while the phosphatidyl residue from lecithin had no effect on the HA activity or the binding of inhibitors (1) We have thus not been able to modify the haemagglutinin so that it is non reactive with inhibitors

How the lipoprotein molecule is organized in the Tween 80-ether extracted (5) rubella haemagglutinin preparation is not known Receptor sites for the inhibitors are no longer exposed on the haemagglutinin when bound to erythrocytes while the antigenic determinants are still free to react with antibody

The new HI procedure proposed here represents an important simplification of the test Attempts at further simplification by using human erythrocytes and thus avoiding the erythrocyte absorption step have not been successful as yet Human erythrocytes are more prone to non specific inhibition than sheep erythrocytes Sheep erythrocytes on the other hand are more readily sensitized for indirect HA than human erythrocytes

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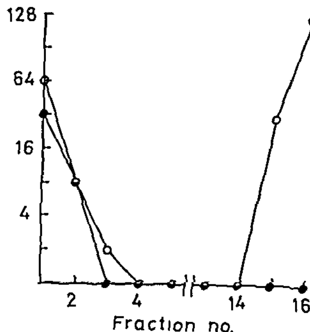


Fig 1 Floatation centrifugation of an HI antibody-positive serum
o——o Standard procedure (except that kaolin treatment is omitted)
•——• *New procedure*

2 One volume of 4 HA units of haemagglutinin is mixed with two volumes of sheep erythrocytes suspended to give an absorbance of 0 8 The mixture is incubated at 4 °C for about 30 min
3 Serum is diluted 1 10 (25 µl of serum and 225 µl of 10% sheep erythrocytes) and shaken in an ice-water bath After centrifugation to *remove the erythrocytes serial two-fold dilu*tions of the serum are made in 25 µl volumes
4 Addition of 75 µl of sensitized erythrocytes Two microtitre plates are usually prepared beforehand during incubation for sensitization ready for the addition of sensitized erythro tes Incubation at 37 °C for 60 min
5 Readings of pattern are made in the us way, after the 60 min incubation at 37 °C after further incubation at 4 °C

Preliminary Results with the New HI Procedure

Floatation centrifugation of an antibody posi serum showed that the new method measu rubella HI antibody but *did not measure n* specific inhibitors (Fig 1)

A panel of 561 sera from the routine mater was examined in parallel using the standard and t new HI procedure (Table 3)

There was relatively good correlation between t two *methods Sera which differed* considerab were re-examined They were prediluted in tube and aliquots of each dilution were transferred to t microtitre wells With this *procedure no differe*ces of more than a one-titre step were observed Th new method was slightly less sensitive than th standard HI method

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Acta path microbiol scand Sect B 87 391–392 1979

BRIEF REPORT

COMPARISON OF TWO SELECTIVE MEDIA IN THE CULTURAL DIAGNOSIS OF GONORRHOEA

Per Leiv Svarva and Johan A Mæland

Department of Microbiology Trondheim Regional Hospital University of Trondheim Trondheim Norway

Svarva P L & Mæland J A Comparison of two selective media in the cultural diagnosis of gonorrhoea Acta path microbiol scand Sect B 87 391–392 1979

A »chocolate« agar medium (CA NCV) containing nystatin colistin and vancomycin was compared with the MNYC medium which contain lincomycin colistin amphotericin and trimethoprim A total of 277 clinical specimens were cultured for gonococci and 120 of these showed positive cultures on either or both of the media The MNYC medium detected 96 6 per cent of the total number of positive cultures and 92 5 per cent of the patients and the CA NCV medium 80 and 79 per cent respectively Nearly half the positive cultures were detected on the MNYC medium after 24 hours of incubation It is concluded that the MNYC medium is superior to the CA NCV medium in the diagnosis of gonorrhoea

Key words Gonorrhoea cultural diagnosis selective media

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Isolation of gonococci by culture is important in the diagnosis of gonorrhoea and many different culture media have been used for this purpose »Chocolate« agar (CA) media are used widely The selective medium of *Thayer & Martin* (5) contains nystatin (N) colistin (C) and vancomycin (V) The isolation rate of gonococci was increased when lincomycin was used instead of vancomycin and trimethoprim lactate was included in the medium (6) In 1973 *Faur et al* (1 2) developed the New York City (NYC) medium and showed that this provided luxuriant growth of gonococci within 24 h of incubation *Young* (7) described modifications of the NYC medium (MNYC) and included lincomycin colistin amphotericin and trimethoprim for selective growth inhibition The MNYC medium was more efficient than the Thayer Martin medium in the diagnosis of gonorrhoea

This study was designed to compare the efficiency of the CA NCV medium used hitherto in our laboratory for the cultural diagnosis of gonorrhoea with that of the MNYC medium

Materials and Methods

A total of 277 specimens from 157 patients 59 women and 98 men were analysed The specimens were obtained from outpatients examined at the Clinic for Venereal Diseases Trondheim Specimens were taken from the urethra cervix and rectum of women and the urethra of men using charcoal impregnated swabs and Stuart transport medium

The CA NCV medium contained GC medium base (Difco) supplemented with one per cent h[illegible]

[illegible] saponin 2 5 per cent (v/v) of a yeast dialysate prepared as described by *Faur et al* (2) 0 02 per [illegible]

met[illegible]

Acta path microbiol scand Sect B 87 393–395 1979

BRIEF REPORT

TOXOPLASMA GONDII SCANNING ELECTRON MICROSCOPE STUDIES ON THE SMALL INTESTINE OF INFECTED AND UNINFECTED CATS

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[1]Biology Department University of Strathclyde George Street Glasgow Scotland [2]Biology Department North East London Polytechnic Romford Road London England and [3]FAO/WHO Collaborating Centre for Research and Reference in Toxoplasmosis Statens Seruminstitut Copenhagen Denmark

Hutchison W M Pittilo R M Ball S J & Siim J Chr *Toxoplasma gondii* scanning electron microscope studies on the small intestine of infected and uninfected cats Acta path microbiol scand Sect B 87 393–395 1979

The mucosal surfaces of villi from the small intestine of cats infected with *Toxoplasma gondii* were studied with the scanning electron microscope and compared with those from uninfected control cats In uninfected cats villi were predominantly leaf shaped and were lined with ridges goblet cell openings could be seen The enterocytes had a hexagonal surface outline and were dome shaped Infected cats had both normal and abnormal villi Injured villi were shortened and attained a broad leaf shape often with blunt edges Enterocytes containing oocysts were enlarged and microvilli were resolvable only on these surfaces Ruptured cells from which parasite discharge had occurred were seen Oocysts were observed and possessed a smooth coat

Key words *Toxoplasma gondii* pathology ultrastructure scanning electron microscopy

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Toxoplasma gondii is a coccidian parasite of the domestic cat within whose small intestine schizogony and gametogony leading to oocyst production occur (5) The advantages of the scanning electron microscope over the dissecting microscope in the study of intestinal mucosae have been adequately demonstrated (1 3 6 10 11) Recently this technique has been used to study damage to the intestinal tract by coccidian parasites of chickens (2 9 12 13 14) and turkeys (7) This report deals with the surface of the intestinal mucosa of the cat and the disruption caused to it by *Toxoplasma*

Materials and Methods

Two strains of *Toxoplasma* were used namely Statens Seruminstitut Strains 119 and 178 both were administered as tissue cysts in brains of experimentally infected mice which had been dye test negative prior to infection Two specific pathogen free (SPF) cats were each fed approximately 75 000 tissue cysts of a mixture of these two strains Two other uninfected SPF cats acted as controls One infected cat was killed at the commencement of oocyst production together with an uninfected control The remaining two cats were sacrificed four days after oocysts could no longer be detected in the faeces of the infected individual Pieces of the small intestine situated 10 cm from the ileocecal [illegible]

[illegible] series then critical point dried After mounting on stubs which were then coated with gold the tissues were examined on a Philips 501 scanning electron microscope

Results

Uninfected controls Villi are long and close packed the majority are leaf shaped (Fig 1) but numerous finger shaped villi are present On the surface of the villi are numerous apertures which correspond to goblet cell openings (Fig 1 & 2) occasionally seen plugged with mucus (Fig 2) Numerous ridges the major ty of [illegible]

TABLE 1 *Clinical Specimens Cultured for* Neisseria gonorrhoeae *on two Different Selective Media with Number and Percentage (in Brackets) of Positive Cultures Obtained after 24 or 48 h of Incubation*

Medium	24	48
CA NCV	26 (21 6)	96 (80)
MNYC	56 (46 6)	116 (96 6)
CA NCV + MNYC	56 (46 6)	120 (100)

composition is different from that reported by *Young* (7) who used 0 1 per cent glucose in the medium

All specimens were plated on each of the culture media on the day taken The media were incubated at 36 5 °C in a candle jar for 24 h and examined for oxidase positive colonies Negative cultures were reexamined after 48 h of incubation Gram negative diplococci were identified by carbohydrate fermentation testing using the fermentation medium described by *Flynn & Waitkins* (3)

Results

Gonococci were isolated from 120 of 277 specimens (Table 1) In all 96 6 per cent of the positive cultures were detected on the MNYC medium and 80 per cent on the CA NCV medium This difference is statistically significant ($p < 0\ 01$ Chi square test) Nearly half the positive cultures could be detected on the MNYC medium after 24 h of incubation which was considerably more than could be detected on the CA NCV medium From 20 specimens gonococci grew on the MNYC medium only and from 4 specimens on the CA NCV medium only Gonorrhoea was diagnosed by culture in 94 patients 87 (92 5 per cent) by use of the MNYC medium and 75 (79 per cent) by the CA NCV medium The MNYC medium detected more positive cultures than the CA NCV medium both in men and women and more positive cultures regardless of the site from which the specimens were taken The two media were about equally effective in suppressing the growth of other bacteria

Discussion

The isolation rate of gonococci on the MNYC medium was 16 6 per cent higher than on the CA NCV medium and 13 5 per cent more of the patients with gonorrhoea were diagnosed by means of the former medium Thus the MNYC medium substantially improved the cultural diagnosis of the disease The results obtained compare favourably with those reported by other investigators (7)

The CA NCV medium contained vancomycin in a concentration of 3 μg/ml that may inhibit the growth of a few strains of gonococci (4) However *Young* (7) showed that strains isolated on the MNYC medium but not on the Thayer Martin medium containing vancomycin (4 μg/ml) usually showed no increased susceptibility to vancomycin The increased isolation rate on the MNYC medium is probably due to a better nutritional value than that of the CA NCV medium This explanation was supported by the observation that many more gonococcal strains showed visible colonies after 24 h of incubation on the MNYC medium than on the CA NCV medium Thus use of the MNYC medium may enable a more rapid cultural diagnosis of the gonorrhoea Unfortunately none of the two media will detect all patients with gonorrhoea If feasible the combined use of culture madia would be preferable

Faur et al (1) reported that glucose showed a stimulating effect on the growth of gonococci up to an addition of 0 6 per cent (w/v) We used only 0 02 per cent of glucose in the MNYC medium This was based on the observation that gonococci showed good growth even with this small quantity of glucose and that growth of contaminating bacteria was much less prominent than with media containing 0 1 per cent glucose as used by *Young* (7)

The optimal concentration of glucose in the MNYC medium used for culture of clinical specimens still remains to be fully elucidated

Since the MNYC medium has advantages over the CA NCV medium in the diagnosis of gonorrhoea we have changed the routine methods in our laboratory accordingly

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tes show a hexagonal outline and are dome shaped in appearance (Fig 2) At high magnifications enterocytes have an uneven surface although separate microvilli were not resolvable

Infected cats Healthy villi are always recognizable but were more abundant in the cat that had ceased oocyst production Injured villi are usually shortened and have a tendency to attain a broad leaf shape often with blunt edges Enterocytes containing a parasite are swollen (Fig 3) often to the extent that separate microvilli can be resolved on the cell surface (Fig 3) After the rupture of enterocytes permitting the escape of parasites apertures are observed with the remains of cell membrane and cytoplasm at the edges (Fig 4) Discharged oocysts possess a smooth surface (Fig 5) Erythrocytes are occasionally observed on surfaces of villi

Discussion

Th

responsible for the fir tree appearance of villi in histological sections (8) and this is also the case with the cat The pronounced ridges seen at the villi tips represent the apical extrusion zone The hexagonal outline of enterocytes is well documented in scanning electron

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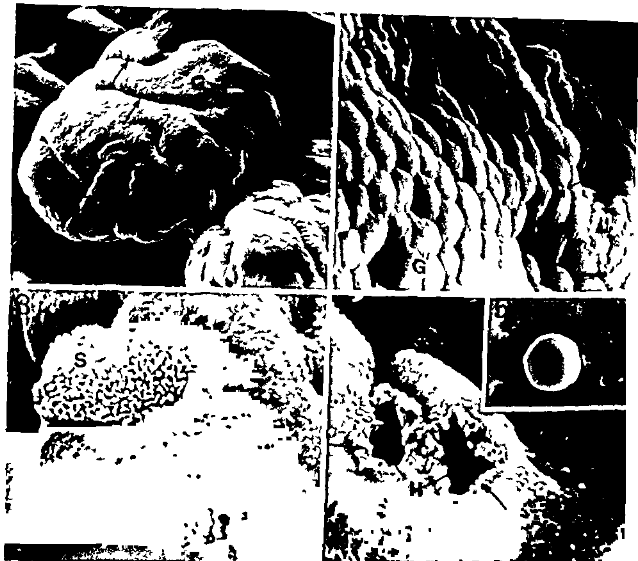
morphological alteration of the mucosa varies greatly The degree of alteration in the cat would appear to be slight in comparison to *Eimeria brunetti* in the chick for example which causes complete destruction of the villi of the ileum (14) Flattened and shortened villi have been reported in *E acervulina* infection of the chick (9 14)

The appearance of shortened villi in infected cats is in agreement with transmission electron microscope studies (4) Both mucus and surface coat are known to prevent resolution of individual microvilli (10) Since the cat intestinal microvilli are known to have a prominent surface coat (6) we did not expect to resolve individual microvilli The fact that we were able to observe individual microvilli in infected enterocytes is probably due to the cell being swollen resulting in the microvilli being separated more than normally In addition the microvilli of enterocytes of *T gondii* infected cats are known to show shortening in relation to parasite distribution in the small intestine (4) and these shortened microvilli may have less adhering mucus after tissue processing The appearance of erythrocytes in the small intestine may be due to slight haemorrhaging but accidental contamination of the tissue with blood may have occurred at autopsy

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We are indebted to Professor *Norman Wright* for the use of scanning electron microscope facilities at the Department of Veterinary Anatomy of Glasgow University The valuable assistance and criticism from Mr *J F Dunachie* and the maintenance of the SPF cats by Mr *J Keys* are gratefully acknowledged Work at Strathclyde University was supported in part by the *W H Ross Foundation* (Scotland) R M P was supported by an *Agricultural Research Council Grant*

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Figures 1 5 are scanning electron micrographs of the intestinal villi of SPF cats Figures 1 and 2 are from uninfected cats and Figures 3 5 are from cats infected with *T gondi*

A single bar (–) on a micrograph represents 2 µm and a double bar ( ) represents 10 µm The following abbreviations are used throughout A ap cal extrusion zone E enterocyte G goblet cell opening H ruptured enterocyte M mucus O oocyst S enlarged enterocytes

F g 1 Leaf shaped villi from an uninfected cat On the villus surface goblet cell openings numerous r dges (arrows) and the apical extrusion zone can be observed × 800

Fig 2 A higher power micrograph of part of a villus s milar to that in Fig 1 Enterocytes can be seen to have a hexagonal outline and to be dome shaped The openings of 3 goblet cells can be seen and a plug of mucus probably obscures a fourth × 3 500

Fig 3 Part of a villus from an infected cat A swollen enterocyte can be seen and ind vidual microvill can just be resolved on its surface × 6 000

Fig 4 Part of villus from an infected cat 2 ruptured enterocytes are shown and the remains of cell membrane and cytoplasm are seen at the aperture edges (arrows) × 6 000

Fig 5 Part of a villus from an infected cat 2 goblet cell openings (arrows) are shown along with a ruptured enterocyte Attached to the villus surface is a *T gond* oocyst × 1 200

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